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Large Expression Differences in Genes for Iron and Zinc Homeostasis, Stress Response, and Lignin Biosynthesis Distinguish Roots of *Arabidopsis thaliana* and the Related Metal Hyperaccumulator *Thlaspi caerulescens*[1][W]

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The micronutrient zinc has an essential role in physiological and metabolic processes in plants as a cofactor or structural element in 300 catalytic and noncatalytic proteins, but it is very toxic when available in elevated amounts. Plants tightly regulate their internal zinc concentrations in a process called zinc homeostasis. The exceptional zinc hyperaccumulator species *Thlaspi caerulescens* can accumulate up to 3% of zinc, but also high amounts of nickel and cadmium, without any sign of toxicity. This should have drastic effects on the zinc homeostasis mechanism. We examined in detail the transcription profiles of roots of *Arabidopsis thaliana* and *T. caerulescens* plants grown under deficient, sufficient, and excess supply of zinc. A total of 608 zinc-responsive genes with at least a 3-fold difference in expression level were detected in *A. thaliana* and 352 in *T. caerulescens* in response to changes in zinc supply. Only 14% of these genes were also zinc responsive in *A. thaliana*. When comparing *A. thaliana* with *T. caerulescens* at each zinc exposure, more than 2,200 genes were significantly differentially expressed (≥5-fold and false discovery rate < 0.05). While a large fraction of these genes are of yet unknown function, many genes with a different expression between *A. thaliana* and *T. caerulescens* appear to function in metal homeostasis, in abiotic stress response, and in lignin biosynthesis. The high expression of lignin biosynthesis genes corresponds to the deposition of lignin in the endodermis, of which there are two layers in *T. caerulescens* roots and only one in *A. thaliana*.

Micronutrients are essential for humans, plants, and animals. The micronutrient zinc plays an important role in physiological and metabolic processes of plants (Ramesh et al., 2004). Zinc serves as a cofactor for more than 300 enzymes, including RNA polymerase, alcohol dehydrogenase, copper/zinc superoxide dismutase, and carbonic anhydrase (Guerinot and Eide, 1999). Zinc is essential but is toxic when available to the plant in elevated amounts; therefore, plants need to keep very tight control over the internal concentrations of zinc in a process called zinc homeostasis.

Although the zinc homeostasis mechanism is supposed to be universal within plants, there are species that can accumulate large amounts of zinc without any sign of toxicity. Species accumulating more than 10,000 μg zinc g^{-1} dry weight (DW; 1% [w/w]) are called zinc hyperaccumulators (Baker and Brooks, 1989). As a comparison, most plants contain between 30 and 100 μg zinc g^{-1} DW and concentrations above 300 μg zinc g^{-1} DW are generally toxic (Marschner, 1995). More than 400 metal hyperaccumulator species from a wide range of unrelated families have been described. About 15 of these are zinc hyperaccumulators (Baker et al., 1992; Brooks, 1994). They are mainly, though not exclusively, found to grow on calamine soils contaminated with lead, zinc, or cadmium (Meerts and Van Isacker, 1997; Schat et al., 2000; Bert et al., 2002). *Thlaspi caerulescens* J. & C. Presl (Brassicaceae) is one of these natural zinc hyperaccumulator species. In addition to zinc, it can also hyperaccumulate cadmium and nickel. It is a self-compatible species, showing variable rates of outcrossing in nature. *T. caerulescens* is closely related to *Arabidopsis thaliana* L. Heynh., with on average 88.5% DNA identity in coding regions (Rigola et al., 2006) and 87% DNA identity in the intergenic transcribed spacer regions (Peer et al., 2003). As in most metal

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hyperaccumulators, the zinc concentration in shoot tissue of *T. caerulescens* is often higher than in root tissue (Lasat et al., 1996; Shen et al., 1997; Schat et al., 2000).

The complex network of homeostatic mechanisms that evolved in plants to control the uptake, accumulation, trafficking, and detoxification of metals (Clemens, 2001) also applies for metal hyperaccumulators. In general, this network involves three major components: transport, chelation, and sequestration. While the physiology of metal hyperaccumulation is already understood fairly well (Clemens et al., 2002), the underlying molecular genetics is still not explored in full detail. Previously published transcript-profiling studies on copper, zinc, and iron deficiency in *A. thaliana* (Wintz et al., 2003) and comparative analysis of *A. thaliana* with the zinc- and cadmium-hyperaccumulating *Arabidopsis halleri* (Becher et al., 2004; Weber et al., 2004) using first generation Affymetrix chips representing a subset of only 8,300 of the approximately 30,000 Arabidopsis genes already identified several genes to respond to zinc deficiency in *A. thaliana*. These analyses also revealed that the transcriptional regulation of many genes is strikingly different in *A. halleri* compared to *A. thaliana*.

In this report, we describe the analysis of three transcript-profiling experiments, with the main aim of establishing which genes are most likely to be relevant for adaptation to high zinc exposure in *T. caerulescens*. Therefore, we examined not only the response of roots of both plant species to zinc deficiency, but also to excess of zinc. We used an Agilent whole-transcriptome, 60-mer oligo DNA microarray representing all annotated genes for *A. thaliana* (further referred to as Arabidopsis) and some 10,000 nonannotated genomic regions with known transcriptional activity, thus covering nearly the complete Arabidopsis transcriptome. In the intraspecific comparison, we identified the Arabidopsis and *T. caerulescens* genes that are differentially expressed a week after transferring the plants to low or high zinc supply. These are relevant in determining differences in the zinc homeostasis network between the two species. We also compared the differences in transcription between the two species at zinc deficiency, sufficiency, and excess supply conditions to identify the genes that are significantly more highly expressed in the hyperaccumulating species compared to Arabidopsis. We finally examined all analyses to identify any particular processes, biochemical pathways, or gene classes that could play a particular role in the adaptation to high zinc accumulation.

RESULTS

Experimental Design

To analyze the response of Arabidopsis and *T. caerulescens* to different zinc exposures, we aimed to compare the transcript profile of plants grown under sufficient zinc supply with plants grown under zinc deficient conditions and excess zinc conditions. To minimize variation in the bioavailability of zinc or other micronutrients, we used a hydroponic rather than soil-based culturing system. For both excess and deficient zinc conditions, the induction of severe stress to the plants was avoided by exposing them for only 1 week to these conditions. Arabidopsis plants (accession Columbia) were established to grow on a nutrient solution containing 2 μM $ZnSO_4$, which is sufficient to yield healthy and robust plants with normal seed set even after prolonged cultivation. After 3 weeks, the plants were transferred to fresh solutions for exposure to zinc deficiency (0 μM $ZnSO_4$) and excess zinc (25 μM $ZnSO_4$). One-third of the plants remained at sufficient zinc (2 μM $ZnSO_4$) as a control. From previous experiments (data not shown), we learned that plants continue to grow in zinc deficient media, while deficiency symptoms (chlorosis and necrosis) or toxicity symptoms become obvious only after about 3 weeks. Upon harvesting root tissue, the plants growing on zinc deficient and sufficient medium did not show any visible phenotypic differences, whereas plants growing on excess zinc showed a slight growth inhibition in the roots (data not shown). At this stage, plants were not flowering yet.

For *T. caerulescens*, a similar approach was taken. However, to properly compare the results between the two species, we aimed at maintaining comparable physiological conditions. *T. caerulescens* accession La Calamine is zinc tolerant as well as zinc hyperaccumulating and requires more zinc than Arabidopsis for normal growth. Therefore, a hydroponic solution containing 100 rather than 2 μM $ZnSO_4$ was used to grow seedlings under zinc sufficient conditions. To avoid any problems with possible precipitation of zinc or other minerals, 1 mM $ZnSO_4$ was used as the excess zinc exposure concentration, although we learned from previous experiments that *T. caerulescens* La Calamine is well able to withstand this exposure for several weeks. When root tissues were harvested after 1-week exposure, the *T. caerulescens* plants showed no altered phenotype that could be attributed to exposure to excess zinc or zinc deficiency.

Mineral Content in Arabidopsis and *T. caerulescens*

Zinc, iron, and manganese concentrations were determined in root and shoots of hydroponically grown Arabidopsis and *T. caerulescens* plants grown at deficient, sufficient, and excess zinc. Comparison of the metal concentration levels between these two species already displayed the typical difference between a metal hyperaccumulator and a metal nonaccumulator (Fig. 1). At low zinc supply (zinc deficiency), the difference is not very pronounced, and although *T. caerulescens* clearly contains more zinc in the leaves, the concentration in the roots for both species is between 1.2 to 1.7 μmol g^{-1} DW (Fig. 1A). At sufficient zinc supply, *T. caerulescens* accumulates about 3 times more zinc in the roots than Arabidopsis, and the concentration in shoots is much higher (approximately

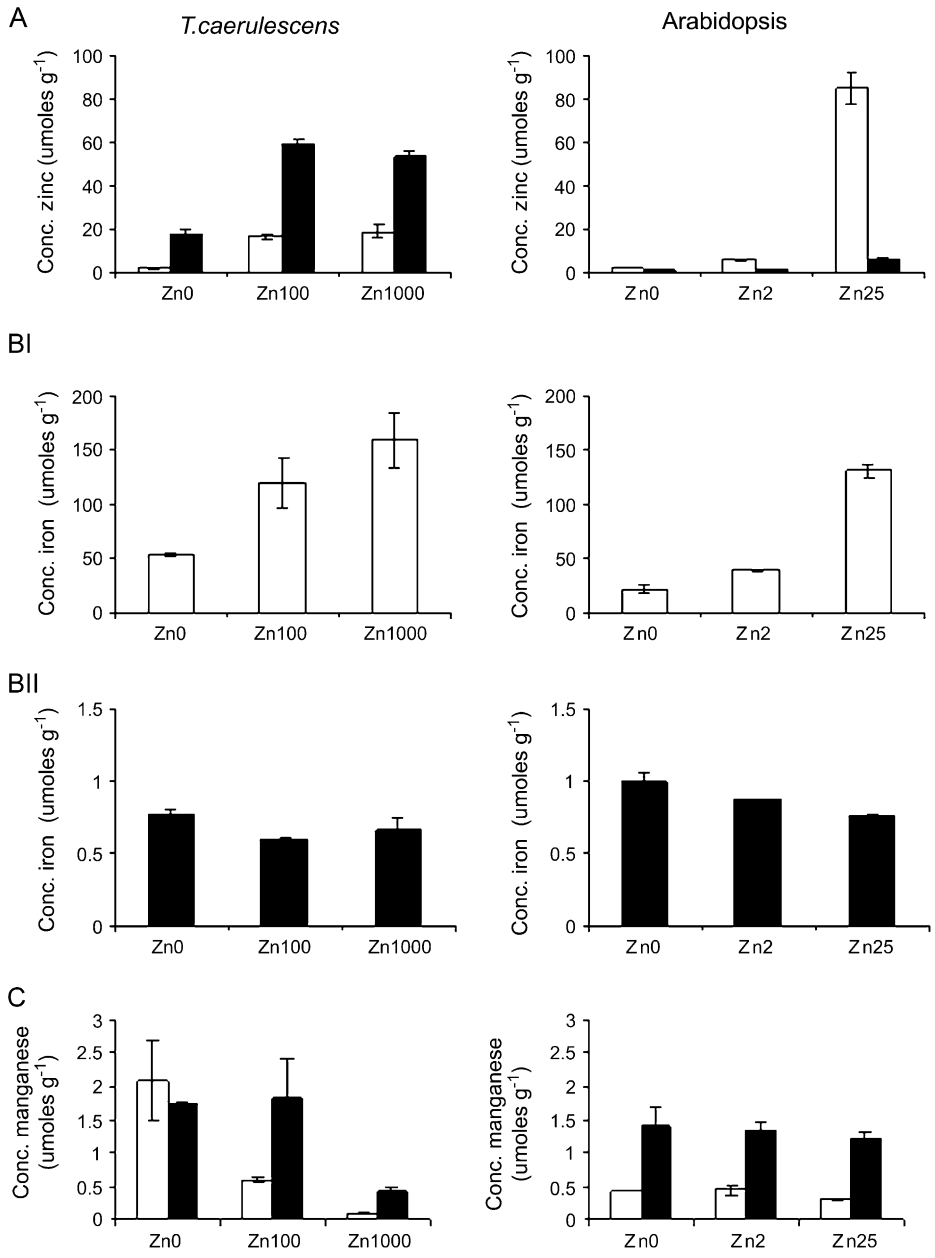

Figure 1. Zinc (A), iron (BI: roots; BII: leaves), and manganese (C) concentrations (μmol g^{-1}; mean ± SE) in Arabidopsis and *T. caerulescens* roots (white bars) and leaves (black bars). Plants were grown for 3 weeks on nutrient solution containing sufficient zinc before exposure to zinc deficiency (0 μM $ZnSO_4$: Zn0), zinc sufficiency (2 or 100 μM $ZnSO_4$: Zn2/Zn100), and excess zinc (25 or 1,000 μM $ZnSO_4$: Zn25/Zn1000).

70-fold) than in Arabidopsis. At excess zinc, the zinc-exclusion strategy of Arabidopsis roots has collapsed and their zinc concentration is now about 4.5-fold higher than in *T. caerulescens*; this is approximately 15-fold higher compared to sufficient conditions in Arabidopsis. At this high zinc supply, Arabidopsis is still able to exclude zinc accumulation in the leaves, with a concentration about 9-fold lower than in *T. caerulescens*. For *T. caerulescens*, there is not much difference in both root and shoot concentrations of plants growing at sufficient or excess zinc.

Since we expected that differences in zinc supply would also affect the concentration of other metals, we measured the iron (Fig. 1B) and manganese (Fig. 1C) concentrations in the same material. Similar to zinc, the iron concentration in the roots of both species increases upon increase in zinc supply (Fig. 1BI). At deficient and sufficient zinc supply, the iron concentration in *T. caerulescens* is about 2- to 3-fold higher than in Arabidopsis. At excess zinc supply, the root iron concentrations are similar for both species. Generally, the iron concentrations are much lower in leaves than in roots. The iron concentration in leaves is similar for the three *T. caerulescens* treatments, and this is only marginally higher in Arabidopsis under sufficient zinc supply (Fig. 1BII). In Arabidopsis leaves, the iron concentration decreases marginally with increasing zinc supply.

For manganese, the situation is the opposite in roots. In *T. caerulescens*, the manganese concentration decreases

with increasing zinc supply and in Arabidopsis this only occurs upon excess zinc supply. The manganese concentration in *T. caerulescens* roots is about 5-fold higher under zinc deficiency than in Arabidopsis, but at sufficient zinc supply there is hardly any difference between the species. There are only few differences for the manganese concentration in leaves between the two species. Only at excess zinc, the manganese concentration in *T. caerulescens* decreases drastically by about 4-fold. Both *T. caerulescens* and Arabidopsis accumulate manganese to a higher concentration in leaves than in roots, with the exception of *T. caerulescens* grown under zinc deficiency.

Zinc Response in Arabidopsis

Genes responding to changes in zinc exposure conditions in Arabidopsis were identified using Agilent Arabidopsis 3 60-mer oligonucleotide microarrays containing 37,683 probes representing more than 27,000 annotated genes and more than 10,000 nonannotated genomic regions for which there is transcriptional evidence. When analyzing the data, we only considered the hybridization data of probes with $P < 0.05$. Only expression differences of ≥3-fold (between any of the three treatments) were considered to be relevant, even though lower expression differences were statistically significant at $P < 0.05$. According to these criteria, we identified 608 zinc-responsive genes when comparing the deficient, sufficient, and excess zinc treatments. As expected, most differences were found between the most distant conditions, zinc deficiency and excess zinc. Many genes that were differentially expressed between zinc deficiency and sufficiency or between zinc sufficiency and excess zinc were also differentially expressed between deficiency and excess zinc, whereas few genes were found to be only differentially expressed between both zinc deficiency and sufficiency, and sufficiency and excess zinc.

After hierarchical clustering (average linkage hierarchical clustering with uncentered correlation; Eisen et al., 1998) of all differentially expressed genes, four major clusters were distinguished. Cluster I (Supplemental Table S1) consists of 98 genes that are more lowly expressed under zinc deficiency compared to sufficient and excess zinc. Within this group, we find many genes with a function related to stress response and also several with metabolism-associated functions. Among the 10 genes most differentially expressed between the zinc deficiency and sufficiency exposures are three genes encoding small heat shock proteins. Other genes found in this cluster are genes encoding copper/zinc superoxide dismutases, a nodulin-like protein, a nitrate-responsive protein, an expansin-like protein, and a universal stress protein. The last one is the most highly expressed gene at sufficient zinc found in this cluster. Fifteen genes in this cluster encode proteins with an unknown function. Twenty probes correspond to transcripts that were not annotated as such in the Arabidopsis genome.

Cluster II (Table I; Supplemental Table S2) is a large cluster consisting of 128 genes. These genes are generally more highly expressed under excess zinc conditions when compared to sufficient or deficient zinc supply. This cluster contains several metal homeostasis-related genes associated with iron rather than with zinc homeostasis. These genes encode metal transporters (*IRT1*, *IRT2*, *ZIP8*, *MTP3*, *MTP8*, *NRAMP4*, and *IREG2*), a nicotianamine (NA) synthase (NAS) gene (*NAS1*), a YS-like oligopeptide transporter (*OPT3*), and ferric-chelate reductases (*FRO1*, *2*, and *3*). Not only are metal homeostasis genes found in this cluster, but also some stress response genes like a disease resistance gene. In addition, metabolic genes like *PAL2* (which encodes a key enzyme acting early in the phenylpropanoid biosynthesis pathway leading to flavonoids, anthocyanins, and lignins) and genes belonging to the cytochrome P450 family (*CYP98A3*, *CYP82C2*, *CYP82C3*, *CYP82C4*, *CYP71B5*, and *CYP71B38*) are found in this cluster. This cluster also contains a small set of genes encoding transcription factors of the basic helix-loop-helix (bHLH), myb, and zinc-finger families.

Cluster III (Table II; Supplemental Table S3) consists of 347 genes that are more highly expressed under zinc deficiency compared to the other two treatments. Many genes in this cluster, especially the ones that show the largest difference in expression between zinc deficiency and excess zinc, belong to metal homeostasis gene families encoding ZIP metal transporters, a cation diffusion facilitator (*MTP* gene family), a P_{IB}-type ATPase transporter (*HMA* gene family), two NAS proteins (*NAS*), a MATE efflux protein (*FRD3*), two ferric-chelate reductase-like proteins (*FRO4* and *5*), ferritin (*FER1*), and two Yellow Stripe1-like proteins (*YSL2* and 3). A surprisingly large fraction of 164 genes encodes proteins without a known function (83) or represent nonannotated transcripts (81). Three of the latter are among the 10 most differentially expressed genes when comparing the three zinc exposure conditions. Other genes identified in this cluster encode proteins involved in protein stability (F-box proteins), signal transduction (calcineurin-like phosphoesterase, auxin response factor, calmodulin-binding proteins, calcium-binding protein, protein kinase), transcriptional regulation (MADS-box, zinc-finger, and bHLH proteins), and metabolism. Among the 10 genes with the highest expression (at sufficient zinc), five encode proteins with an unknown function (At5g19380, At5g16870, At2g16990, At3g15630, and At4g29905), one encodes a nonannotated transcript, and three are involved in transcriptional regulation (At1g72220, At3g01970, and At3g51080). Of the 10 most differentially expressed genes, *FRO5*, *MTP2*, *NAS4*, and *IRT3* have a supposed role in metal homeostasis based on their predicted function or their similarity to other genes previously implicated in metal homeostasis. Only the superoxide dismutase is remarkably differentially expressed between all three treatments, decreasing in expression upon increase of the zinc concentration in the medium.

Table I. *Arabidopsis genes more highly expressed under excess (Zn25) zinc conditions when compared to sufficient (Zn2) or deficient (Zn0) zinc supply*

Name	Code[a]	Putative Function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Intensity[d]
SAM1	*At1g02500*	*S-Adenosylmethionine synthetase 1*	*Other cellular, metabolic, physiological processes*	*0.98*	*0.31*	*0.31*	*62,719*
GLP5	*At1g09560*	*Germin-like protein*	*Biological process unknown*	*0.64*	*0.11*	*0.18*	*27,296*
	At1g73120	*Expressed protein*	*Biological process unknown*	*0.85*	*0.30*	*0.35*	*13,274*
FRO2	*At1g01580*	*Ferric-chelate reductase*	*Transport*	*0.27*	***0.02***	***0.06***	*12,522*
PAL2	*At3g53260*	*Phenylalanine ammonia-lyase 2*	*Response to abiotic or biotic stimulus*	*1.00*	*0.28*	*0.28*	*12,379*
	At1g74760	*Zinc-finger (C3HC4-type RING finger) family protein*	*Protein metabolism*	*0.70*	*0.20*	*0.29*	*10,028*
	At4g13860	*Gly-rich RNA-binding protein*	*Biological process unknown*	*0.39*	*0.15*	*0.38*	*7,988*
	At1g63090	*F-box family protein*	*Protein metabolism*	*0.39*	*0.11*	*0.30*	*7,941*
	At5g45080	*Disease resistance protein related*	*Response to abiotic or biotic stimulus*	*0.62*	*0.24*	*0.39*	*7,650*
	At3g53480	*ABC transporter family protein*	*Biological process unknown*	*0.74*	*0.28*	*0.38*	*7,391*
NAS1	At5g04950	Nicotianamine synthase	Other cellular, metabolic, physiological processes	2.63	0.62	0.24	4,791
IREG2	At5g03570	Iron-responsive transporter	Biological process unknown	1.25	0.27	0.22	3,592
MTP3	At3g58810	Zinc transporter	Transport	0.68	0.04	0.06	3,178
ATNRAMP4	At5g67330	NRAMP metal ion transporter 4	Transport	0.69	0.32	0.46	2,644
FRO3	At1g23020	Ferric-chelate reductase	Electron transport or energy pathways	0.87	0.18	0.21	2,559
ZIP8	At5g45105	Metal transporter	Transport	0.33	**0.02**	**0.06**	2,099
	At3g12900	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	Biological process unknown	0.09	**0.00**	**0.04**	1,937
FRO1	At1g01590	Ferric-chelate reductase	Electron transport or energy pathways	0.23	0.04	0.16	1,659
IRT1	At4g19690	Iron-responsive transporter	Transport	0.35	**0.02**	**0.06**	1,530
OPT3	At4g16370	Oligopeptide transporter OPT family protein	Transport	0.39	0.12	0.31	931
IRT2	At4g19680	Iron-responsive transporter	Transport	0.38	**0.01**	0.04	832
bHLH100	At2g41240	bHLH family protein	Transcription	0.21	1.00	**0.02**	572
CYP82C3	At4g31950	Cytochrome P450 family protein	Electron transport or energy pathways	0.18	**0.01**	0.08	527
CYP82C2	At4g31970	Cytochrome P450 family protein	Electron transport or energy pathways	0.21	**0.01**	0.05	476
CYP82C4	At4g31940	Cytochrome P450 family protein	Electron transport or energy pathways	0.14	**0.00**	**0.03**	389
MYB72	At1g56160	Myb family transcription factor	Transcription	0.37	**0.01**	**0.03**	99
MTP8	At3g58060	Cation efflux family protein	Transport	0.45	0.05	0.11	87

[a]AGI gene code (At…). [b]GO annotations according to biological process. [c]Ratio of significant (FDR $P < 0.05$) differential (≥3) expressed genes between two zinc exposure conditions. Zn0 = 0 μM $ZnSO_4$; Zn2 = 2 μM $ZnSO_4$; Zn25 = 25 μM $ZnSO_4$. [d]Normalized spot intensity at 2 μM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. The 10 probes with highest hybridization intensity at 2 μM $ZnSO_4$ are in italic. Ten probes with highest ratios for Zn0/Zn25 and Zn2/Zn25 comparisons are in bold.

Cluster IV (Supplemental Table S4) consists of 35 genes that show a lower expression under excess zinc exposure compared to deficient and sufficient zinc exposures. Genes in this cluster are involved in (secondary) metabolism, (a)biotic stress response, and transcription. Five genes in this cluster encode proteins with an unknown function. Nine genes were not annotated.

When comparing all three zinc exposure conditions, only genes encoding ferric-chelate reductases (*FRO1* and *5*), two ZIP metal transporters (*ZIP3* and *9*), iron superoxide dismutase (*FSD1*), an oxidoreductase (At3g12900), an iron-sulfur cluster assembly complex protein (At2g36260), cytochrome P450 CYP82C4, and an expressed protein (At3g59930) are differentially

Table II. *Arabidopsis genes more highly expressed under zinc deficiency (Zn0) compared to sufficient (Zn2) and excess (Zn25) zinc supply*

Name	Code[a]	Putative Function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Intensity[d]
	At1g72220	*Zinc-finger (C3HC4-type RING finger) family protein*	*Protein metabolism*	*1.36*	*3.35*	*2.46*	*83,438*
	At5g19380	*Expressed protein*	*Biological process unknown*	*2.41*	*3.23*	*1.34*	*48,547*
	At5g16870	*Expressed protein*	*Biological process unknown*	*2.36*	*4.44*	*1.88*	*37,115*
	At2g16990	*Expressed protein*	*Biological process unknown*	*2.46*	*3.21*	*1.30*	*34,791*
	At1g72500	*Inter-α-trypsin inhibitor heavy chain related*	*Biological process unknown*	*2.73*	*3.33*	*1.22*	*30,230*
	At3g15630	*Expressed protein*	*Biological process unknown*	*3.05*	*4.46*	*1.46*	*30,117*
	At4g29905	*Expressed protein*	*Biological process unknown*	*3.98*	*7.83*	*1.97*	*29,854*
WRKY45	*At3g01970*	*WRKY family transcription factor*	*Transcription*	*2.22*	*3.30*	*1.49*	*26,487*
	At3g51080	*Zinc-finger (GATA-type) family protein*	*Transcription*	*3.31*	*2.70*	*0.82*	*26,114*
	CHR5:20218751–20219267	*Unknown*	*Biological process unknown*	*2.93*	*3.43*	*1.17*	*25,354*
FSD1	At4g25100	Superoxide dismutase (iron)	Response to abiotic or biotic stimulus	5.31	**76.80**	**14.47**	22,269
ATFER1	At5g01600	Ferritin 1	Response to abiotic or biotic stimulus, transport	1.82	5.18	2.85	14,350
ZIP3	At2g32270	Zinc transporter	Transport	6.10	31.45	5.16	7,477
ZIP2	At5g59520	Zinc transporter	Transport	1.23	13.45	10.95	6,366
NAS2	At5g56080	Nicotianamine synthase	Other cellular, metabolic, physiological processes	4.56	1.77	0.39	4,994
	At5g50400	Calcineurin-like phosphoesterase family protein	Biological process unknown	10.45	13.83	1.32	4,460
FRO4	At5g23980	Ferric-chelate reductase	Electron transport or energy pathways	3.02	14.47	4.79	4,331
YSL3	At5g53550	Transporter	Transport	1.88	4.10	2.18	3,633
	CHR2:15208700–15208384	Unknown	Biological process unknown	**75.17**	**616.09**	8.19	2,897
ZIP5	At1g05300	Metal transporter	Transport	5.75	22.53	3.92	1,484
	At1g20380	Prolyl oligopeptidase	Protein metabolism	**42.64**	**106.37**	2.49	1,368
ZIP4	At1g10970	Metal transporter	Transport	15.03	36.71	2.44	1,263
FRO5	At5g23990	Ferric-chelate reductase	Electron transport or energy pathways	9.75	**105.49**	10.82	867
ZIP11	At1g55910	Metal transporter	Transport	3.61	4.00	1.11	717
	CHR4:6931720–6932736	Unknown	Biological process unknown	**135.58**	**917.14**	6.76	702
ZIP1	At3g12750	Zinc transporter	Transport	9.37	15.64	1.67	681
YSL2	At5g24380	Transporter	Transport, response to abiotic or biotic stimulus	1.95	3.35	1.72	635
NAS4	At1g56430	Nicotianamine synthase	Other cellular, metabolic, physiological processes	**44.32**	18.11	0.41	630
HMA2	At4g30110	ATPase E1-E2-type family protein	Transport	6.48	7.44	1.15	628
ZIP9	At4g33020	Metal transporter	Transport	35.88	8.94	0.25	617
	At2g36260	Iron-sulfur cluster assembly complex protein	Biological process unknown	22.63	**117.05**	5.17	533
FRD3	At3g08040	MATE efflux family protein	Other cellular, metabolic, physiological processes	5.63	7.71	1.37	483
IRT3	At1g60960	Metal transporter	Transport	22.71	**90.01**	3.96	435
	At1g71200	bHLH family protein	Transcription	4.37	8.60	1.97	431
MTP2	At3g61940	Zinc transporter	Transport	**175.34**	**214.38**	1.22	152
ZIP10	At1g31260	Metal transporter	Transport	7.46	9.47	1.27	136
	CHR2:15209769–15208753	Unknown	Biological process unknown	**92.35**	**157.48**	1.70	66
ZIP12	At5g62160	Metal transporter	Transport	9.23	12.48	1.35	58

[a]AGI gene code (At…) or chromosome position of nonannotated transcript (CHRX:…). [b]GO annotations according to biological process. [c]Ratio of significant (FDR $P < 0.05$) differential (≥3) expressed genes between two zinc exposure conditions. Zn0 = 0 μM $ZnSO_4$; Zn2 = 2 μM $ZnSO_4$; Zn25 = 25 μM $ZnSO_4$. [d]Normalized spot intensity at 2 μM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. The 10 probes with highest hybridization intensity at 2 μM $ZnSO_4$ are in italic. Ten probes with highest ratios in Zn0/Zn2 and Zn0/Zn25 comparisons are in bold.

expressed between all conditions (Tables I and II; Supplemental Table S3).

Heterologous Microarray Hybridization

We used the same Arabidopsis array platform for heterologous hybridization with labeled *T. caerulescens* cDNA. From analysis of approximately 3,500 expressed sequence tags (ESTs), we previously determined that *T. caerulescens* shares about 85% to 90% DNA identity in coding regions with Arabidopsis (Rigola et al., 2006). However, since most probes present on the arrays were designed to fit less conserved regions of the Arabidopsis transcripts, we verified the suitability of cross-species hybridization of these arrays. First, Agilent Arabidopsis 1 oligonucleotide arrays, representing around 13,500 putative genes, were hybridized with labeled cDNA from Arabidopsis and *T. caerulescens* roots grown under sufficient zinc conditions. The spot intensities of the *T. caerulescens* hybridizations were on average only 1.7-fold lower than the spot intensities of Arabidopsis hybridizations, which is sufficient for reliable expression analysis. In addition, genomic DNA hybridization of *T. caerulescens* to the Agilent Arabidopsis 3 oligonucleotide array showed average 2.0-fold lower signal intensity for *T. caerulescens* compared to the Arabidopsis signal intensities. Overall, only probes corresponding to 220 genes did not hybridize with *T. caerulescens* genomic DNA (less than 3-fold less signal intensity). These 220 genes were excluded from the dataset.

Zinc Response in *T. caerulescens*

When comparing the expression of genes in roots of *T. caerulescens* plants grown on deficient, sufficient, and excess zinc media, we identified 350 genes that were significantly (false discovery rate [FDR] $P < 0.05$) differentially expressed (≥3-fold) in any of the three possible comparisons. Only 50 of these were also differentially expressed in response to different zinc exposures in Arabidopsis. Six clusters were identified in this set upon cluster analysis (average linkage hierarchical clustering with uncentered correlation). Clusters I and II (Table III; Supplemental Table S5) consist of 38 genes that are more highly expressed at zinc deficiency compared to sufficient or excess zinc treatments. ZIP-like genes *ZIP3*, *ZIP4*, and *ZIP9* are coexpressed, showing higher expression at sufficient zinc compared to excess zinc. This in contrast to the *ZIP1* and *ZIP2* genes, which are expressed at similar levels under zinc sufficiency and excess zinc. Other known metal homeostasis genes found in this cluster are the *NAS4* and *FRO5* genes. These were also found to be more highly expressed in zinc deficient Arabidopsis roots. The *FSD1* iron superoxide dismutase, which

Table III. *T. caerulescens genes more highly expressed under deficient (Zn0) compared to sufficient (Zn100) and excess (Zn1000) zinc supply*

Cluster	Name	Code[a]	Putative Function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Intensity[d]
I	ZIP4	AT1G10970.1	Metal transporter	Transport	2.47	4.73	1.91	47,323
	FSD1	AT4G25100.1	Iron superoxide dismutase	Circadian rhythm	5.89	**30.04**	5.10	6,430
	ZIP3	AT2G32270.1	Zinc transporter	Transport	2.22	5.38	2.43	2,151
	ZIP9	AT4G33020.1	Metal transporter	Transport	1.95	4.43	2.27	1,472
II	CYP83A1	AT4G13770.1	Cytochrome P450 family protein	Response to abiotic or biotic stimulus	**6.70**	4.33	0.65	7,900
		AT1G20380.1	Prolyl oligopeptidase, putative	Protein metabolism	**7.63**	4.95	0.65	4,007
	ZIP1	AT3G12750.1	Zinc transporter	Transport	**9.33**	**10.11**	1.08	1,569
	ZIP2	AT5G59520.1	Zinc transporter	Transport	4.48	3.49	0.78	1,539
	NAS4	AT1G56430.1	Nicotianamine synthase	Other cellular, metabolic, physiological processes	4.99	3.55	0.71	1,432
	ERD9	AT1G10370.1	Glutathione *S*-transferase	Other cellular, metabolic, physiological processes	**18.83**	**8.17**	0.43	902
	FRO5	AT5G23990.1	Ferric-chelate reductase	Electron transport or energy pathways	**8.47**	**7.10**	0.84	391
		AT1G75260.1	Isoflavone reductase family protein	Biological process unknown	**9.42**	4.53	0.48	278
		AT5G06730.1	Peroxidase	Response to abiotic or biotic stimulus	**8.69**	5.41	0.62	276
	CA2	AT5G14740.1	Carbonic anhydrase 2	Other metabolic processes	**50.67**	**40.39**	0.80	93
	DIR5	AT1G64160.1	Disease resistance-responsive family protein	Response to abiotic or biotic stimulus	**124.24**	**67.00**	0.54	82

[a]AGI gene code (At…). [b]GO annotations according to biological process. [c]Ratio of significant (FDR $P < 0.05$) differential (≥3) expressed genes between two zinc exposure conditions. Zn0 = 0 μM $ZnSO_4$; Zn100 = 100 μM $ZnSO_4$; Zn1000 = 1,000 μM $ZnSO_4$. [d]Normalized spot intensity at 100 μM $ZnSO_4$. Genes are ordered according to decreasing spot intensity at 100 μM $ZnSO_4$. Ten probes with highest ratios for Zn0/Zn100 and Zn0/Zn1000 comparisons are presented in bold.

was also found to be differentially expressed in Arabidopsis, is found in this cluster. The most differentially expressed gene encodes a Dirigent protein (*DIR5*), involved in lignin biosynthesis. This gene is hardly expressed under sufficient zinc conditions, which explains the strong differential expression.

Clusters IIIA and IIIB (Supplemental Table S6) consist of 74 and 16 genes, respectively, more highly expressed under deficient and excess zinc conditions compared to sufficient zinc. Genes in cluster IIIA are predominantly more highly expressed under zinc deficiency; genes in cluster IIIB are predominantly more highly expressed under excess zinc. Many genes associated with oxidative stress response, senescence, ethylene biosynthesis, and plant defense are found in these clusters, including genes encoding peroxidases and four plant defensin fusion genes (*PDF1.1*, *PDF1.2b*, *PDF1.2c*, and *PDF1.3*). There are 11 genes with an unknown function in this cluster, of which one is not annotated.

Clusters IVA and IVB (Supplemental Table S7) consist of 19 and 14 genes, respectively, all most highly expressed under excess zinc compared to the other two conditions. Two of these genes (At5g05250 and At2g41240) are also found in a similar cluster for Arabidopsis roots (Table I; Supplemental Table S2). Compared to Arabidopsis, the Thlaspi cluster is much smaller and lacks all of the iron homeostasis genes.

The remaining 189 genes fall into two additional clusters, generally more highly expressed under sufficient than under deficient conditions (Supplemental Tables S8 and S9). Almost half of these encode proteins with an unknown function. Many of the other genes are involved in general metabolism and stress response.

Difference in Zinc Response between Arabidopsis and *T. caerulescens*

To identify genes that may be crucial for the adaptive differences between Arabidopsis and *T. caerulescens*, we compared the gene expression profiles between the two species for each of the tested physiological conditions. Taking into account that we performed a heterologous hybridization and that probes generally did not hybridize as efficiently to *T. caerulescen*s cDNA as to Arabidopsis cDNA, we only considered significant probes with a more than 5-fold higher normalized hybridization signal in *T. caerulescens* compared to Arabidopsis in any of the three comparisons to be of biological relevance.

According to these criteria, in total 2,272 genes were found to be at least 5 times significantly more highly (FDR $P < 0.05$) expressed in *T. caerulescens* compared to Arabidopsis (Supplemental Table S10). Of these genes, 420 (18.5%) were not found to be expressed in roots of Arabidopsis under comparable conditions (Supplemental Table S11). A large class of 1,147 of the 2,272 differentially expressed genes has an unknown biological function. Other classes represent genes encoding proteins involved in cellular processes, transport processes, stress response, and transcription. A total of 929 genes showed little variation in expression under the three tested conditions, suggesting a constitutively higher expression in *T. caerulescens* roots. To test if this expression is anyhow functionally related to metal stress adaptation, the functional distribution of this group was compared to that of all 2,272 differentially expressed genes, but this did not show specific gene classes to be overrepresented or underrepresented (data not shown). Only 121 of the 2,272 genes are differentially expressed in *T. caerulescens* in response to different zinc exposures. The most highly expressed genes among these are the *ZIP4* and *IRT3* metal transporters (Tables III and IV). Remarkable is the large difference in expression between *T. caerulescens* and Arabidopsis of four members of the *PDF* gene family (Table IV; Supplemental Table S12). These genes are especially highly expressed in *T. caerulescens* under deficient and excess zinc conditions compared to Arabidopsis. To facilitate the further analysis of this large class of genes, a selection was made of 235 genes, including the 50 most differentially expressed genes under zinc deficiency and genes of which the proposed function could be relevant in explaining the metal adaptation differences between both species (Table IV; Supplemental Table S12).

Next to putative metal homeostasis genes and stress response genes, several genes suggested to be involved in lignin biosynthesis (Ehlting et al., 2005) also were much more highly expressed in *T. caerulescens* compared to Arabidopsis. In addition to the higher expression of the lignin biosynthesis genes, genes potentially involved in suberin biosynthesis (*CER3*, *CER6*, and 11 *LTP*-genes) also were more highly expressed in *T. caerulescens*, though not as high as the lignin biosynthesis genes (Table IV; Supplemental Table S12). Out of 24 genes putatively involved in lignin biosynthesis, 11 were generally more than 10-fold higher differentially expressed and four were among the 15 highest expressors when absolute expression levels at sufficient zinc were considered (Table IV; Supplemental Table S12). This higher expression was expected to cause visible differences in lignification between *T. caerulescens* and Arabidopsis roots. To identify such differences, transverse sections of 4-, 6-, and 9-week-old roots from *T. caerulescens* and 4- and 6-week-old roots from Arabidopsis plants grown hydroponically at sufficient zinc supply were made and examined by UV microscopy at wavelengths that induce lignin and suberin autofluorescence. After 4 weeks, Arabidopsis roots only showed autofluorescence of the xylem and the outer wall of epidermis cells in sections taken 2 cm from the root tip (Fig. 2A). At this age, *T. caerulescens* roots also showed autofluorescence of the xylem vessels, and the inner wall of the endodermis cells also lighted up (Fig. 2, D and E). Epidermal fluorescence was not seen in *T. caerulescens*. Sections of older *T. caerulescens* root parts, more distant from the tip, showed stronger fluorescence of, especially, the endodermis

Table IV. *A selection of 109 genes more highly expressed in T. caerulescens (Tc) compared to Arabidopsis (At)*

Name	Code[a]	Putative Function	GO Annotation[b]	Tc/At			Tc			At			Intensity[d]
				Zn0/ Zn0[c]	Zn100/ Zn2[c]	Zn1000/ Zn25[c]	Zn0/ Zn100[c]	Zn0/ Zn1000[c]	Zn100/ Zn1000[c]	Zn0/ Zn2[c]	Zn0/ Zn25[c]	Zn2/ Zn25[c]	
PDF1.1	At1g75830	Plant defensin-fusion protein	Response to stress	948.50	10.17	717.66	8.38	**0.65**	0.08	0.09	**0.49**	5.49	12,363
	At4g02280	Suc synthase	Metabolism; Suc biosynthesis	597.18	131.02	1,369.28	**2.48**	**0.46**	**0.18**	**0.54**	**1.05**	**1.93**	20,639
RPL13C	*At3g48960*	*60S ribosomal protein L13*	*Protein biosynthesis*	*365.86*	*167.78*	*202.29*	***1.04***	***0.99***	***0.96***	***0.47***	***0.55***	***1.16***	*47,849*
	At5g43935	Flavonol synthase	Metabolism; flavonol synthesis	330.48	112.84	211.01	**1.38**	**1.31**	**0.95**	0.47	**0.84**	**1.78**	5,242
	At4g28005	Expressed protein	Electron transport; iron ion binding	280.19	123.06	216.14	**1.01**	**1.07**	**1.05**	**0.45**	**0.82**	**1.84**	10,503
PDF1.2b	At2g26020	Plant defensin-fusion protein	Response to stress	260.94	18.41	522.12	7.22	**0.59**	0.08	**0.51**	**1.19**	**2.33**	3,264
	At5g28510	*Ferredoxin hydrogenase protein*	*Carbohydrate metabolism*	*219.05*	*103.40*	*180.24*	***1.19***	***1.34***	***1.13***	***0.56***	***1.10***	***1.97***	*60,950*
	CHR4:11881512–11882528	Unknown	Biological process unknown	201.76	144.61	315.46	**0.56**	**0.43**	**0.76**	**0.40**	**0.67**	**1.66**	25,676
	At1g17710	Expressed protein	Metabolism; thiamine biosynthesis	180.12	26.33	115.19	**2.99**	**0.71**	**0.24**	**0.44**	**0.46**	**1.04**	1,583
PDF1.3	At2g26010	Plant defensin-fusion protein	Response to stress	166.01	7.64	284.86	6.34	**0.50**	0.08	0.29	**0.86**	**2.95**	11,977
	At2g16200	Hypothetical protein	Biological process unknown	161.28	87.95	178.28	**1.07**	**1.23**	**1.15**	**0.58**	**1.35**	**2.33**	10,725
	At3g50270	Transferase family protein	Response to stress	153.69	30.87	113.21	**2.83**	**2.07**	**0.73**	**0.57**	**1.53**	**2.69**	3,598
DIR13	*At4g11190*	*dis. res.-responsive family protein*	*Metabolism; lignin biosynthesis*	*149.04*	*114.62*	*124.81*	***1.16***	***1.16***	***1.00***	***0.89***	***0.97***	***1.09***	*135,715*
PDF1.2c	At5g44430	Plant defensin-fusion protein	Response to stress	139.17	8.83	323.60	6.48	**0.50**	0.08	**0.41**	**1.17**	**2.85**	11,917
APX2	At3g09640	L-Ascorbate peroxidase 1b	Response to stress	137.11	54.53	169.21	**1.42**	**0.87**	**0.61**	**0.57**	**1.08**	**1.91**	33,325
HAK5	At4g13420	Potassium transporter	Cation transport	121.37	36.31	124.20	**1.16**	**0.83**	**0.72**	0.35	**0.85**	2.47	14,613
	CHR4:9566304–9565288	Unknown	Biological process unknown	121.07	138.45	117.38	**0.52**	**0.50**	**0.95**	**0.60**	**0.48**	**0.81**	4,470
	At5g41820	Geranylgeranyl transferase	Protein metabolism	120.48	104.47	72.61	**0.51**	**0.45**	**0.88**	**0.44**	0.27	**0.61**	9,054
	At5g55430	Hypothetical protein	Biological process unknown	116.78	52.60	27.51	**1.52**	**1.32**	**0.87**	**0.68**	**0.31**	**0.45**	7,203
	At1g26360	Hypothetical protein	Metabolism; biotin biosynthesis	111.19	98.83	166.66	**1.13**	**1.04**	**0.92**	**1.00**	**1.56**	**1.55**	5,549
	At3g13784	β-Fructosidase	Metabolism; carbohydrate metabolism	104.90	84.25	31.02	**1.18**	**1.06**	**0.89**	**0.95**	**0.31**	**0.33**	2,545
	CHR2:5739298–5739397	Unknown	Biological process unknown	103.32	111.21	176.21	**0.58**	**0.44**	**0.76**	**0.62**	**0.75**	**1.21**	22,083
	At1g06030	pfkB-type carbohydrate kin. prot.	D-Rib metabolism	101.24	46.43	129.15	**1.42**	**0.95**	**0.67**	**0.65**	**1.22**	**1.87**	26,682
	At1g14960	*Major latex protein related*	*Unknown; defense related*	*98.69*	*66.29*	*71.91*	***1.06***	***1.08***	***1.02***	***0.71***	***0.78***	***1.11***	*89,031*
	At4g21950	Hypothetical protein	Biological process unknown	98.10	50.35	30.94	**1.12**	**0.79**	**0.71**	**0.57**	**0.25**	**0.43**	1,744
	At3g59590	Jacalin lectin family protein	Biological process unknown	96.47	76.25	48.03	**0.88**	**1.12**	**1.27**	**0.70**	**0.56**	**0.80**	14,688
	At2g16490	XH domain-containing protein	Biological process unknown	96.25	98.11	220.05	**0.47**	**0.38**	**0.80**	**0.48**	**0.86**	**1.80**	20,789
	At4g23150	Protein kinase family protein	Protein metabolism	94.01	88.21	220.21	**0.53**	**0.46**	**0.87**	**0.50**	**1.08**	**2.17**	8,278
PHT3	At5g43360	Inorganic phosphate transporter	Phosphate transport	93.26	38.32	78.99	**1.77**	**1.23**	**0.70**	**0.73**	**1.04**	**1.44**	19,836
	At1g49250	ATP-dependent DNA ligase protein	DNA repair	91.87	50.56	31.91	**1.34**	**1.19**	**0.89**	**0.74**	**0.41**	**0.56**	2,284
	CHR5:19326437–19325921	Unknown	Biological process unknown	91.69	106.55	132.71	**0.48**	**0.41**	**0.84**	**0.56**	**0.59**	**1.05**	10,162

(*Table continues on following page.*)

Table IV. (*Continued from previous page.*)

Name	Code[a]	Putative Function	GO Annotation[b]	Tc/At			Tc			At			Intensity[d]
				Zn0/ Zn0[c]	Zn100/ Zn2[c]	Zn1000/ Zn25[c]	Zn0/ Zn100[c]	Zn0/ Zn1000[c]	Zn100/ Zn1000[c]	Zn0/ Zn2[c]	Zn0/ Zn25[c]	Zn2/ Zn25[c]	
	CHR5:26539519–26539835	Unknown	Biological process unknown	91.58	128.49	148.44	**0.51**	**0.42**	**0.82**	**0.71**	**0.67**	**0.95**	15,584
	At1g62690	Expressed protein	Biological process unknown	91.31	46.75	68.49	**1.01**	**0.89**	**0.89**	**0.51**	**0.67**	**1.30**	2,325
	CHR3:23273840–23274356	Unknown	Biological process unknown	87.80	96.98	208.78	**0.60**	**0.43**	**0.71**	**0.66**	**1.01**	**1.53**	14,196
	At1g40087	Hypothetical protein	Biological process unknown	87.50	41.06	56.51	**1.41**	**1.20**	**0.85**	**0.66**	**0.77**	**1.17**	1,915
	CHR2:7453552–7452536	Unknown	Biological process unknown	87.17	84.55	441.91	**0.52**	**0.40**	**0.76**	**0.51**	**2.01**	3.97	28,488
	At2g38250	DNA-binding protein	Transcription	84.05	57.36	120.09	**0.54**	**0.40**	**0.76**	0.37	**0.58**	**1.58**	23,033
	At2g21330	*Fru-bisphosphate aldolase*	*Metabolism*	*78.66*	*32.23*	*73.24*	***1.77***	***1.04***	***0.59***	***0.72***	***0.96***	***1.33***	*91,786*
	At5g58330	Malate dehydrogenase	Metabolism	77.13	54.94	107.84	**0.62**	**0.48**	**0.77**	**0.44**	**0.67**	**1.52**	7,882
	At2g35740	Sugar transporter family protein	Transport	75.23	81.52	143.47	**0.56**	**0.43**	**0.76**	**0.60**	**0.81**	**1.34**	11,976
	CHR4:6561567–6560402	Unknown	Biological process unknown	73.91	119.35	209.56	**0.51**	**0.42**	**0.83**	**0.83**	**1.20**	**1.45**	7,686
	At2g30830	2-Oxoglutarate-dependent dioxygenase	Metabolism	71.27	17.04	19.01	2.49	**2.14**	**0.86**	**0.60**	**0.57**	**0.96**	5,756
	CHR1:13243304–13243548	Unknown	Biological process unknown	70.40	35.47	141.84	**1.61**	**0.41**	**0.26**	**0.81**	**0.83**	**1.02**	1,853
	CHR1:11026453–11026996	Unknown	Biological process unknown	70.20	101.45	231.20	**1.29**	**1.31**	**1.02**	**1.38**	**2.50**	**1.81**	199
	At4g28750	PSI reaction center subunit IV	Photosynthesis	69.01	41.98	227.92	**0.55**	**0.42**	**0.77**	**0.33**	**1.39**	4.19	15,894
	CHR3:7075276–7075592	Unknown	Biological process unknown	68.68	66.19	26.01	**0.95**	**1.07**	**1.12**	**0.92**	**0.40**	**0.44**	2,637
4CLL8	At5g38120	4-Coumarate-CoA ligase family protein	Metabolism; lignin biosynthesis	68.48	60.18	53.69	**0.69**	**0.96**	**1.39**	**0.61**	**0.75**	**1.24**	5,574
	At5g37980	NADP-dependent oxidoreductase	Response to abiotic or biotic stimulus	68.30	63.11	71.61	**0.68**	**0.55**	**0.81**	**0.63**	**0.58**	**0.92**	12,423
GA4H	At1g80340	GA 3-β-dioxygenase	Metabolism; GA biosynthesis	67.35	16.56	23.69	4.79	4.08	**0.85**	**1.18**	**1.44**	**1.22**	1,155
	At3g50940	AAA-type ATPase family protein	Biological process unknown	66.42	46.58	54.31	**1.01**	**0.83**	**0.82**	**0.71**	**0.68**	**0.95**	2,803
LTP4	At5g59310	Lipid transfer protein 4	Transport	47.97	48.95	52.56	**0.51**	**1.02**	**1.98**	**0.52**	**1.12**	**2.13**	5,979
4CLL3	At1g20500	4-Coumarate-CoA ligase family	Metabolism; lignin biosynthesis	47.67	45.86	53.39	**0.65**	**0.90**	**1.39**	**0.62**	**1.01**	**1.62**	3,956
MTP8	At3g58060	Cation efflux family protein	Cation transport	43.33	29.35	3.05	**0.67**	**0.71**	**1.06**	**0.45**	0.05	0.11	2,693
KAT1	At5g46240	Inward-rectifying potassium channel	Cation transport	40.06	34.09	40.41	**1.10**	**1.15**	**1.05**	**0.93**	**1.16**	**1.24**	4,106
MT2A	*At3g09390*	*Metallothionein protein*	*Response to stress; copper binding*	*32.47*	*20.25*	*84.39*	***1.40***	***0.51***	*0.37*	***0.87***	***1.33***	***1.52***	*131,218*
4CLL2	At1g20490	AMP-dependent synthetase/ligase	Metabolism; lignin biosynthesis	27.24	37.52	26.07	**0.79**	**0.94**	**1.19**	**1.08**	**0.90**	**0.83**	15,214
	At1g73550	Lipid transfer protein (LTP) family protein	Transport	24.68	13.12	10.12	**1.09**	**0.96**	**0.88**	**0.58**	0.39	**0.68**	13,278
NRAMP3	At2g23150	NRAMP metal ion transporter 3	Cation transport	23.97	7.86	18.56	2.07	**0.89**	0.43	**0.68**	**0.69**	**1.02**	18,082
DIR11	At1g22900	Disease resistance-responsive fam. prot.	Metabolism; lignin biosynthesis	23.47	17.99	14.33	**1.10**	**1.44**	**1.31**	**0.84**	**0.88**	**1.04**	3,974
CCR2	At1g80820	Cinnamyl-CoA reductase	Metabolism; lignin biosynthesis	22.03	10.81	10.86	**1.24**	**1.60**	**1.29**	**0.61**	**0.79**	**1.30**	22,963
DIR23	At3g13660	dis. res.-response protein related	Metabolism; lignin biosynthesis	17.36	52.11	16.87	**0.52**	**1.32**	**2.56**	**1.55**	**1.29**	**0.83**	2,860
CCRL14	*At5g19440*	*Cinnamyl-alcohol dehydrogenase*	*Metabolism; lignin biosynthesis*	*17.20*	*9.07*	*11.55*	***1.03***	***1.07***	***1.04***	*0.54*	***0.72***	***1.32***	*98,965*
HMA4	*At2g19110*	*ATPase E1-E2-type family protein*	*Cation transport*	*16.41*	*11.15*	*15.36*	***1.73***	***0.96***	***0.56***	***1.18***	***0.90***	***0.77***	*105,390*

(*Table continues on following page.*)

Table IV. (*Continued from previous page.*)

Name	Code[a]	Putative Function	GO Annotation[b]	Tc/At			Tc			At			Intensity[d]
				Zn0/ Zn0[c]	Zn100/ Zn2[c]	Zn1000/ Zn25[c]	Zn0/ Zn100[c]	Zn0/ Zn1000[c]	Zn100/ Zn1000[c]	Zn0/ Zn2[c]	Zn0/ Zn25[c]	Zn2/ Zn25[c]	
IRT3	At1g60960	Metal transporter	Cation transport	13.82	123.65	281.56	**2.54**	**4.42**	**1.74**	22.71	90.04	**3.96**	42,981
bHLH100	At2g41240	bHLH fam. prot.	Transcription	13.62	**3.24**	**0.59**	**0.89**	0.09	0.10	**0.21**	0.00	0.02	2,195
CAX7	At5g17860	Cation exchanger	Cation transport	12.79	7.33	13.12	**1.69**	**0.90**	**0.53**	**0.97**	**0.92**	**0.95**	850
DIR20	At1g58170	dis. res.-responsive protein related	Metabolism; lignin biosynthesis	11.93	7.22	6.05	**2.15**	**2.30**	**1.07**	**1.30**	**1.17**	**0.90**	2,774
	At1g62500	Lipid transfer protein (LTP) family protein	Transport	11.85	6.77	12.80	**1.22**	**1.13**	**0.93**	**0.70**	**1.22**	**1.75**	7,639
CADL5	At4g39330	Mannitol dehydrogenase	Metabolism; lignin biosynthesis	10.12	15.81	11.44	0.46	**0.82**	**1.79**	**0.72**	**0.93**	**1.30**	14,903
KUP3	At3g02050	Potassium transporter	Cation transport	9.69	9.38	9.58	**1.12**	**1.11**	**0.99**	**1.08**	**1.09**	**1.01**	3,395
CER3	At5g02310	Eceriferum3 protein	Transcription; zinc binding	9.54	9.31	11.15	**1.20**	**1.03**	**0.86**	**1.17**	**1.20**	**1.03**	5,275
FAH1	*At4g36220*	*Cyt. P450 84A1 ferulate-5 hydroxylase*	*Metabolism; lignin biosynthesis*	*9.27*	*22.78*	*18.70*	***0.63***	***0.77***	***1.23***	***1.54***	***1.56***	***1.01***	*95,951*
CCRL3	At1g09500	Cinnamyl-alcohol dehydrogenase family	Metabolism; lignin biosynthesis	8.79	3.65	8.81	2.60	**1.25**	**0.48**	**1.08**	**1.25**	**1.15**	1,147
GLP11c	At3g04180	Germin-like protein	Metabolism; lignin biosynthesis	8.78	6.75	8.16	**1.13**	**0.51**	**0.46**	**0.87**	**0.48**	**0.55**	355
COMTL8	At1g63140	*O*-Methyltransferase	Metabolism; lignin biosynthesis	8.15	14.35	6.42	**0.62**	**1.05**	**1.71**	**1.09**	**0.83**	**0.76**	636
CAX9	At3g14070	Cation exchanger	Cation transport	8.03	3.53	4.89	**1.17**	**0.96**	**0.83**	0.51	**0.59**	**1.15**	1,454
ZIP10	At1g31260	Metal transporter	Cation transport	7.66	33.02	17.24	**1.73**	**4.20**	**2.43**	**7.46**	9.47	**1.27**	5,104
CCRL13	At5g14700	Cinnamyl-CoA reductase related	Metabolism; lignin biosynthesis	7.63	**1.33**	**1.15**	4.84	5.01	**1.04**	**0.84**	**0.75**	**0.89**	531
	At3g22120	Lipid transfer protein (LTP) family prot.	Transport	7.17	6.07	8.20	**1.02**	**1.59**	**1.56**	**0.86**	**1.82**	**2.11**	16,632
CCOMTL1	At1g67980	Caffeoyl-CoA 3-*O*-methyltransferase	Metabolism; lignin biosynthesis	7.10	**0.83**	8.45	3.64	**1.01**	0.28	**0.42**	**1.20**	**2.83**	1,191
LTP3	At5g59320	Lipid transfer protein 3	Other physiological processes	7.01	18.79	15.18	0.52	**1.08**	2.09	**1.39**	2.35	**1.69**	11,235
	At4g00165	Lipid transfer protein (LTP) family protein	Transport	6.98	13.36	7.89	**0.50**	**0.93**	**1.86**	**0.95**	**1.05**	**1.10**	9,474
	At5g48490	*Lipid transfer protein (LTP) family protein*	*Transport*	*6.31*	*4.75*	*7.23*	***1.29***	***0.93***	***0.72***	***0.97***	***1.07***	***1.10***	*108,087*
CAD1	*At4g34230*	*Cinnamyl-alcohol dehydrogenase*	*Metabolism; lignin biosynthesis*	*6.22*	*5.96*	*5.05*	***1.27***	***1.19***	***0.94***	***1.22***	***0.97***	***0.79***	*64,332*
HMA3	At4g30120	ATPase E1-E2-type family protein	Cation transport	6.05	5.70	1.98	**0.98**	**1.20**	**1.23**	**0.92**	0.39	**0.43**	5,882
CADL1	At1g72680	Cinnamyl-alcohol dehydrogenase	Metabolism; lignin biosynthesis	5.85	5.17	6.06	**0.82**	**0.79**	**0.97**	**0.72**	**0.82**	**1.14**	13,341
COMTL4	At1g21130	*O*-Methyltransferase	Metabolism; lignin biosynthesis	5.79	**3.27**	8.12	**1.76**	**1.36**	**0.78**	**0.99**	**1.91**	**1.93**	7,465
YSL7	At1g65730	Oligopeptide transporter OPT fam. prot.	Cation transport	5.76	3.43	6.95	**1.43**	**0.81**	**0.57**	**0.85**	**0.98**	**1.15**	1,121
IRT2	At4g19680	Iron-responsive transporter	Cation transport	5.72	**1.17**	0.06	**1.83**	**1.37**	**0.75**	**0.38**	0.01	0.04	962
CLA3	*At2g27250*	*CLAVATA3 CLAVATA3/ESR related*	*Signal transduction; development*	*5.56*	*14.73*	*6.84*	***0.40***	***1.27***	***3.20***	***1.05***	***1.56***	***1.49***	*48,437*
	At1g73780	Lipid transfer protein (LTP) family protein	Transport	5.48	7.98	4.49	**0.65**	**1.31**	2.00	**0.95**	**1.07**	**1.12**	14,883
ZIP4	*At1g10970*	*Metal transporter*	*Cation transport*	*4.88*	*29.66*	*37.86*	*2.47*	*4.73*	***1.91***	*15.03*	*36.71*	*2.44*	*47,323*
CER3	At5g02300	Eceriferum3 protein	Transcription; zinc binding	4.87	15.64	24.65	**0.57**	**0.40**	**0.70**	**1.83**	**2.02**	**1.10**	29,407
CCRL9	At2g23910	Cinnamyl-CoA reductase related	Metabolism; lignin biosynthesis	4.83	6.46	5.13	**0.53**	**0.71**	**1.34**	**0.71**	**0.76**	**1.07**	2,244

(*Table continues on following page.*)

Table IV. (*Continued from previous page.*)

Name	Code[a]	Putative Function	GO Annotation[b]	Tc/At			Tc			At			Intensity[d]
				Zn0/ Zn0[c]	Zn100/ Zn2[c]	Zn1000/ Zn25[c]	Zn0/ Zn100[c]	Zn0/ Zn1000[c]	Zn100/ Zn1000[c]	Zn0/ Zn2[c]	Zn0/ Zn25[c]	Zn2/ Zn25[c]	
4CLL9	At5g63380	4-Coumarate-CoA ligase family protein	Metabolism; lignin biosynthesis	4.73	3.83	5.73	**1.06**	**0.80**	**0.75**	**0.86**	**0.97**	**1.12**	4,488
LTP2	At2g38530	Nonspecific lipid transfer protein 2	Other physiological processes	4.58	6.23	5.44	**0.72**	**1.05**	**1.46**	**0.98**	**1.24**	**1.27**	39,419
MT-2B	*At5g02380*	*Metallothionein protein 2B*	*Response to stress; copper binding*	*4.38*	*2.30*	*6.50*	***1.72***	***0.66***	*0.38*	***0.90***	***0.97***	***1.08***	*164,846*
	At4g22460	Lipid transfer protein (LTP) family protein	Transport	4.25	16.23	4.82	**0.46**	**0.92**	**1.99**	**1.77**	**1.05**	**0.59**	21,693
LAC07	At3g09220	Laccase family protein	Metabolism; lignin biosynthesis	3.85	4.20	5.20	**0.95**	**0.96**	**1.01**	**1.04**	**1.30**	**1.26**	27,502
CER6	At1g68530	Very-long-chain fatty acid cond. enzyme	Metabolism	3.58	11.07	5.34	**0.43**	**1.26**	2.92	**1.33**	**1.88**	**1.41**	2,808
FER4	At2g40300	Ferritin	Iron ion homeostasis	3.50	6.72	5.20	**0.92**	**1.45**	**1.58**	**1.77**	2.16	**1.22**	3,330
MTP1	*At2g46800*	*Zinc transporter*	*Cation transport*	*3.33*	*4.66*	*5.62*	***0.64***	***0.60***	***0.94***	***0.89***	***1.01***	***1.13***	*67,213*
FRD3	At3g08040	MATE efflux family protein	Iron ion homeostasis	3.08	11.86	23.52	**1.46**	**1.01**	**0.69**	5.63	7.71	**1.37**	6,335
4CL1	At1g51680	4-Coumarate-CoA ligase 1	Metabolism; lignin biosynthesis	3.05	5.23	**0.99**	**1.01**	**1.29**	**1.28**	**1.73**	0.42	0.24	28,495
	At3g43720	Lipid transfer protein (LTP) family protein	Transport	2.84	6.24	6.13	0.53	**0.86**	**1.61**	**1.17**	**1.86**	**1.58**	4,113
AE6	At5g16340	AMP-binding protein	Metabolism; lignin biosynthesis	2.70	3.66	5.55	**0.70**	**0.60**	**0.85**	**0.95**	**1.23**	**1.29**	5,392
NAS2	At5g56080	Nicotianamine synthase	Electron transport or energy pathways	**1.22**	6.47	2.96	**0.86**	**0.73**	**0.85**	4.56	**1.77**	0.39	41,042
ZIP5	At1g05300	Metal transporter	Cation transport	**0.93**	**2.34**	6.50	**2.29**	**3.23**	**1.41**	5.75	22.54	**3.92**	4,438
FRO5	At5g23990	Ferric-chelate reductase	Electron transport or energy pathways	**0.43**	**0.49**	6.32	8.47	**7.10**	**0.84**	9.75	105.46	10.82	391

Included are the 50 most differentially expressed genes under zinc deficiency and genes encoding putative metal homeostasis-related transporters, lignin biosynthesis proteins, transcription factors, and signal transduction proteins. [a]AGI gene code (At…) or chromosome position of nonannotated transcript (CHRX:…). [b]GO annotations according to biological process. [c]Ratio of significant (FDR $P < 0.05$) differential (≥3 or 5) expressed genes between two zinc exposure conditions. Values are ratios based on the average log ratios of two biological replicates. Zn0 = 0 μM $ZnSO_4$; Zn2 = 2 μM $ZnSO_4$; Zn25 = 25 μM $ZnSO_4$; Zn100 = 100 μM $ZnSO_4$; Zn1000 = 1,000 μM ZnSO4. [d]Normalized spot intensity at 100 μM $ZnSO_4$. Fifteen genes with highest normalized spot intensity at 100 μM $ZnSO_4$ are in italic. Genes not significantly differentially expressed are in bold ($P \geq 0.05$).

cells compared to the younger parts. In Arabidopsis, this staining was much weaker (Fig. 2, B and C). The formation of a second layer with endodermis-like fluorescent staining was observed in the older Thlaspi roots (Fig. 2, F and G) but never in Arabidopsis roots (Fig. 2, B and C).

Semiquantitative Reverse Transcription-PCR

For confirmation of the microarray expression profiling data, a small subset consisting of differentially expressed genes and random genes was subjected to semiquantitative reverse transcription (RT)-PCR. In the absence of *T. caerulescens* DNA sequences suitable for designing species-specific PCR primers, orthologous *T. caerulescens* gene fragments were first amplified by low-stringency PCR using Arabidopsis-specific primers and confirmed by DNA sequencing. This sequence was used to design primers for semiquantitative RT-PCR hybridizing at comparable positions of *T. caerulescens* and Arabidopsis gene sequences. Expression of the target genes was studied in both root and leaf tissues of plants grown hydroponically at different zinc supply conditions (Fig. 3). In general, the root expression levels determined by semiquantitative RT-PCR were comparable to those determined by microarray analysis, confirming the significance of the heterologous microarray hybridization results.

When considering the expression in leaves, there are some striking differences between Arabidopsis and *T. caerulescens* that could not be observed in the root microarray comparison. First of all, the expression of three of the four *NAS* genes is different between the two species. *AtNAS1* is predominantly expressed in roots in Arabidopsis. In contrast, *TcNAS1* only shows detectable expression in leaves of *T. caerulescens*. *AtNAS3* is mainly expressed under zinc deficiency in both roots and leaves of Arabidopsis. In *T. caerulescens*, the *TcNAS3* gene is much more strongly expressed in leaves than in roots, with only a slightly higher expression at lower zinc supply levels. In Arabidopsis,

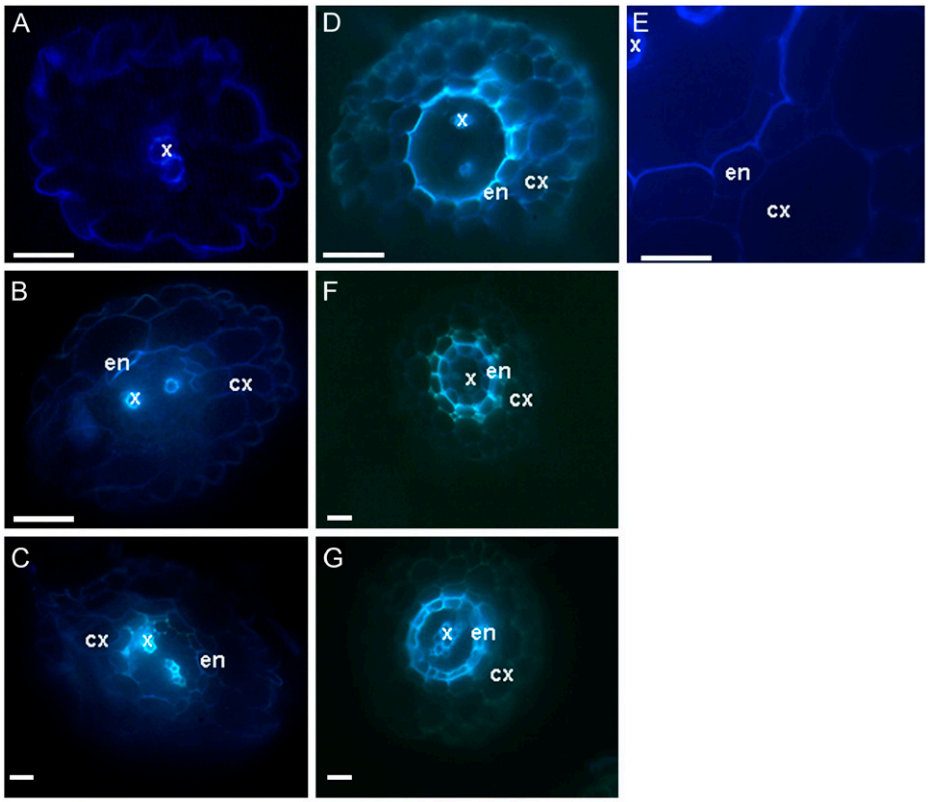

Figure 2. UV autofluorescence of lignin and suberin deposition in comparable cross sections of roots of hydroponically grown Arabidopsis (A, B, and C) and *T. caerulescens* (D, E, F, and G). A, Cross section made 2 cm from the tip of a 4-week-old Arabidopsis root showing blue UV fluorescence of the xylem vessels and the epidermis outer cell walls. B, Cross section made 2 cm from the root tip of a 6-week-old Arabidopsis root showing strong UV fluorescence of the xylem vessels and light fluorescence of the inner wall of endodermis cells. C, Cross section made 6 cm from the root tip of a 6-week-old Arabidopsis root showing strong UV fluorescence of the xylem and faint fluorescence of the inner wall of the endodermis cells. D, Cross section made 2 cm from the tip of a 4-week-old *T. caerulescens* root showing strong UV fluorescence of the xylem vessels and the inner walls of the endodermis cells. E, Close-up of a cross section made 1 cm from the root tip of a 4-week-old *T. caerulescens* root showing the UV fluorescence of the xylem vessels and the inner walls of the endodermis cells. F, Cross section made 6 cm from the root tip of a 6-week-old *T. caerulescens* root showing UV fluorescence of the xylem vessels and the inner or outer walls of endodermis cells. Remarkable is the apparent formation of a second layer of endodermis cells, of which the inner walls are also fluorescent. G, Cross section of a 9-week-old *T. caerulescens* root made 6 cm from the root tip showing two layers of endodermis, both of which show UV fluorescence. cx, Cortex; en, endodermis; x, xylem. Bar = 20 μm.

AtNAS4 is induced by zinc deficiency in roots and zinc excess in leaves. The *TcNAS4* in *T. caerulescens* does not show zinc-responsive expression and is constitutively expressed in the roots and leaves. Of four *NAS* genes, only *AtNAS2* and *TcNAS2* show comparable expression in both species.

When comparing the other differentially expressed genes, *TcAPX2*, *TcHMA4*, and *TcZIP4 (ZNT1)* are all constitutively expressed in *T. caerulescens* leaves. Expression of these genes was either not detected in Arabidopsis leaves (*APX2* and *HMA4*) or only detected at zinc deficiency conditions (*ZIP4*). Also, the expression of *FER1* and *FRO4* in leaves differs between the two species: *TcFER1* is induced under zinc excess conditions while *AtFER1* is induced under low zinc concentrations, and *TcFRO4* is not expressed in the leaves while *AtFRO4* is slightly induced at sufficient and excess zinc conditions. The *HAK5* potassium transporter and the *CSD1* copper/zinc superoxide dismutase genes show similar expression in leaves and roots of both species. In Arabidopsis, the At1g20380 gene (encoding a putative prolyl oligopeptidase) is strongly induced at low zinc conditions, whereas the *T. caerulescens* ortholog is more or less constitutively expressed at low levels in roots and leaves. *ZIP1* is also constitutively lowly expressed in *T. caerulescens* roots and leaves, but induced in Arabidopsis roots at zinc deficiency. Furthermore, we found the *IRT1* iron transporter gene to be very differently expressed in Arabidopsis compared to *T. caerulescens*. *AtIRT1* is induced by excess zinc in Arabidopsis roots. For *TcIRT1*, this induction is much weaker in *T. caerulescens* roots, and the overall expression levels also are much lower than for the *AtIRT1* gene, confirming the observed absence of this gene from Thlaspi cluster IV (Supplemental Table S7).

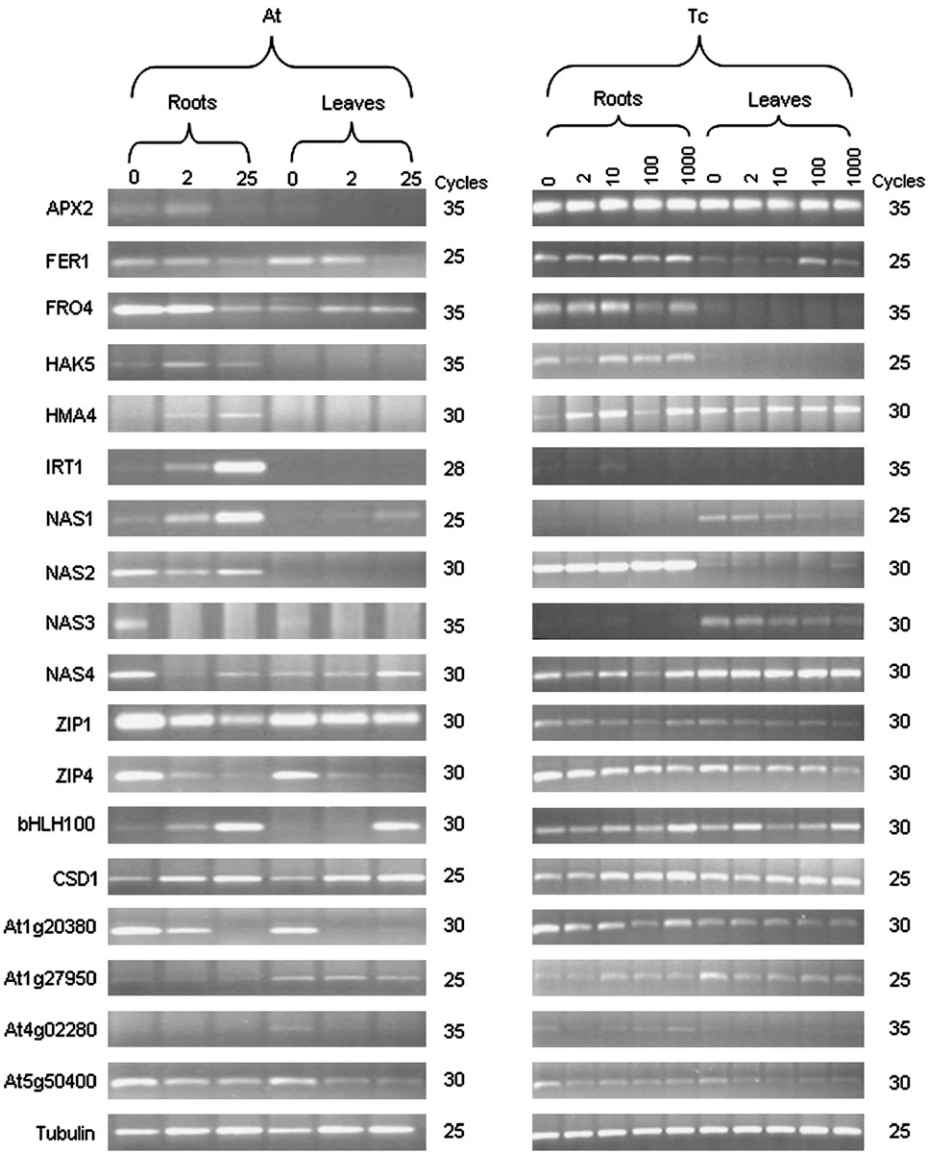

Figure 3. Comparative semiquantitative RT-PCR of selected putative metal homeostasis-related differentially expressed genes (APX2 to At1g20380) and three randomly picked genes (At1g27950 to At5g50400) in Arabidopsis (At) and *T. caerulescens* (Tc). For amplification, species-specific primers were designed at comparable locations in each orthologous gene pair. Roots and leaves were harvested separately after 1 week of exposure of 3-week-old plants to 0, 2, and 25 μM $ZnSO_4$ for Arabidopsis and 0, 2, 10, 100, and 1,000 μM $ZnSO_4$ for *T. caerulescens*. APX2, L-ascorbate peroxidase, At3g09640; FER1, ferritin, At5g01600; FRO4, ferric chelate reductase-like, At5g23980; HAK5, potassium transporter, At4g13420; HMA4, zinc ATPase E1-E2 type, At2g19110; IRT1, Fe(II) transporter, At4g19690; NAS1, nicotianamine synthase, At5g04950; NAS2, nicotianamine synthase, At5g56080; NAS3, nicotianamine synthase, At1g09240; NAS4, nicotianamine synthase, At1g56430; ZIP1, zinc transporter, At3g12750; ZIP4, zinc transporter, At1g10970; bHLH100, bHLH transcription factor, At2g41240; CSD1, copper/zinc superoxide dismutase, At1g08830; putative prolyl oligopeptidase, At1g20380; lipid transfer protein related, At1g27950; Suc synthase, At4g02280; calcineurin-like phosphoesterase, At5g50400. Tubulin (At1g04820) was used as a control for equal cDNA use.

DISCUSSION

In this study, we investigated the expression of genes in roots of the nonaccumulator species Arabidopsis in response to exposure to three very different zinc concentrations in hydroponic culture. We postulate that genes that show differential expression under different zinc exposures are most likely to be involved in metal homeostasis. Most of these will be differentially expressed as a consequence of downstream changes in the physiological status of plants due to changes in metal homeostasis, but a few genes will be directly involved in regulating metal homeostasis. In trying to identify the latter ones, we also examined the differential expression of genes in the zinc hyperaccumulator *T. caerulescens*. Of the three metals we tested, only zinc homeostasis is clearly different between the two species. While Arabidopsis is not able to maintain nontoxic zinc levels in roots upon exposure to excess zinc levels in the nutrient solution (Fig. 1A; Becher et al., 2004; Talke et al., 2006), *T. caerulescens* is perfectly able to do this even while translocating high amounts of zinc to the leaves (Fig. 1A; Assunção et al., 2003b). After only 1 week, the zinc content in *T. caerulescens* is not as high as previously found by Assunção et al. (2003a), who measured after several weeks of exposure. Unexpectedly, iron accumulates in the roots of both *T. caerulescens* and Arabidopsis at increasing zinc concentrations (Fig. 1BI). Based on absence of an effect of iron status on zinc uptake in *T. caerulescens* (Lombi et al., 2002) and the antagonistic effect found for Arabidopsis seedlings (Thomine et al., 2003), we expected no effect or an antagonistic effect of the zinc status on iron uptake. The synergistic effect we found suggests that both species may increase their iron uptake as a response to a possible risk of iron deficiency

in leaves. If so, this strategy is effective since no actual decrease is seen in iron concentration in *T. caerulescens* leaves and only a slight decrease is observed in Arabidopsis leaves (Fig. 1BII). For manganese, there is an antagonistic response of decreased uptake upon increased zinc uptake in the roots (Fig. 1C).

When examining gene expression in the same material of both species, we expected that genes that are differentially expressed between the two species, and especially those that show a difference in response to changes in the external zinc concentration, may be crucial to the adaptive difference between a zinc accumulator and a nonaccumulator. Previously, aspects of the metal accumulator versus nonaccumulator gene expression comparison have been studied for Arabidopsis and *A. halleri* (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006). In addition to those studies, we compared and verified root gene expression under three different zinc exposure conditions (deficient, sufficient, and excess zinc) that clearly lead to different zinc concentrations in roots and leaves of both species. Another important addition to previous studies is that the Agilent 3 oligo microarray we used contains approximately 40,000 probes representing more than 27,000 annotated genes and more than 10,000 nonannotated transcripts (http://www.chem.agilent.com) and is thus an almost complete representation of the Arabidopsis transcriptome. We propose that genes that are induced in expression upon transfer to zinc deficiency or upon transfer to excess zinc are most interesting for further understanding of zinc homeostasis in Arabidopsis. Among the first class are some genes already known to be involved in zinc homeostasis, such as *ZIP2*, *4*, *5*, and *9*, *NAS2*, and *HMA2* genes (Grotz et al., 1998; Wintz et al., 2003; Talke et al., 2006). In addition, we confirmed previous suggestions of genes to be involved in zinc homeostasis, such as *ZIP1*, *3*, and *10*, *IRT3*, *MTP2*, and *NAS4*, to be more highly expressed under zinc deficiency. *ZIP1*, *NAS2*, and *NAS4* were also induced in *A. halleri* in response to low zinc supply (Becher et al., 2004). Our results now suggest there are at least 10 different members of the *ZIP* gene family (Guerinot and Eide, 1999) that play a role in zinc uptake in roots (*ZIP1*, *2*, *3*, *4*, *5*, *9*, *10*, *11*, and *12*, and *IRT3*). Hypothesizing that these transporters are involved in transport of cations across the plasma membrane, it is unlikely that all of them are involved in the uptake of zinc in the same tissue. Most likely, these transporters exert a similar function in different parts of the root or are located in intracellular membranes.

Of the other two known zinc transporters induced by zinc deficiency, *HMA2* has been implicated in transport of zinc into the vasculature, either to promote zinc export from root to shoot via the xylem or from shoot to root via the phloem (Eren and Argüello, 2004; Hussain et al., 2004). In the first case, higher expression under zinc deficiency could be a response to a higher zinc demand from the shoot; in the latter, it could be to accommodate the higher zinc demand of the root by remobilizing zinc from the shoot. The very strong induction of *MTP2* is remarkable. Rather than *MTP1* (previously known as *ZAT*), which is constitutively expressed in Arabidopsis (van der Zaal et al., 1999; Kobae et al., 2004), the induction of *MTP2* by zinc deficiency suggests a specific role of this transporter in counteracting the effect of zinc deficiency.

Metals are often chelated in planta to NA. The absence of NA has severe effects on metal homeostasis, as was observed in the *chloronerva* mutant of tomato (*Lycopersicon esculentum*; Ling et al., 1999) or in NA metabolizing NAAT-overexpressing tobacco (*Nicotiana tabacum*) plants (Takahashi et al., 2003). NA is formed by trimerization of *S*-adenosylmethionine catalyzed by the enzyme NAS. Arabidopsis contains four *NAS* genes, at least three of which are able to catalyze the last step in the synthesis of NA (Suzuki et al., 1999; Becher et al., 2004; Weber et al., 2004). Only *NAS2* and *NAS4* are more highly expressed in roots under zinc deficiency compared to sufficiency (Fig. 3A), but the presence of several, apparently paralogous *NAS* genes with different overlapping gene expression profiles suggests complementary and possibly redundant functions.

In addition to the higher expression of *NAS* genes, some *YSL* genes also are induced by zinc deficiency. These genes are implicated in transport of metal-NA chelates within the plant (Curie et al., 2001; Waters et al., 2006) and possibly the entry of metals into the phloem or xylem (DiDonato et al., 2004). We find expression of *YSL2* and *YSL3* to be only slightly affected by different zinc treatments, and contrary to the observations by Schaaf et al. (2005), we find the genes to be slightly induced by lower zinc concentrations. Recently, Waters et al. (2006) also showed there is an induction of *YSL3* in Arabidopsis grown under zinc deficiency conditions. Unexpected was the high zinc deficiency-induced expression of *FRD3*, *FRO4*, and *FRO5*. Although the *frd3* mutant has a zinc accumulation phenotype, *FRD3* has been mainly implicated in iron homeostasis (Lahner et al., 2003; Green and Rogers, 2004). *FRO4* and *FRO5* resemble the ferric chelate reductase gene *FRO2* (Robinson et al., 1999), but, in contrast to *FRO2*, their expression is not induced in Arabidopsis roots upon iron deficiency (Mukherjee et al., 2005; Wu et al., 2005). The current results suggest a much broader role in general metal homeostasis of these genes than previously thought.

In addition to these genes, we identified 328 other probes with a similar differential transcription profile (Table II; Supplemental Table S3), indicating a similar involvement in zinc homeostasis for the corresponding genes (Eisen et al., 1998). For some of these probes, the only indication of a corresponding gene came from massive parallel sequence signature analysis (Meyers et al., 2004), as no Arabidopsis gene was annotated at that position. Three of these are among the 10 most differentially expressed when comparing zinc deficient and sufficient conditions. So far we have not identified the corresponding genes. For three other

genes in this cluster, a prolyl oligopeptidase (At1g20380), a calcineurin-like phosphoesterase (At5g50400), and a bHLH family protein (At1g71200), knockout (KO) mutants were examined but not found to display any aberrant phenotype under differential zinc exposure (data not shown). Prolyl oligopeptidase and calcineurin-like phosphoesterase need metals, possibly also zinc, to function properly. The same holds for the iron-sulfur cluster assembly protein and carbonic anhydrase 1. This can explain their zinc-responsive expression profile.

Another large cluster of 128 differentially expressed genes is more highly expressed upon exposure to zinc excess. Expression of many of these appears to be associated with the defense against oxidative stress caused by this treatment (e.g. peroxidase, respiratory burst oxidase proteins). This cluster also comprises genes of families that are associated with iron deficiency response, such as *ZIP* genes (*IRT1*, *IRT2*, *ZIP8*), *FRO* genes (*FRO1*, *2*, *3*), *MTP*s (*MTP3*, *8*), a *NAS* gene (*NAS1*), an oligopeptide transporter (*OPT3*), and *IREG2*. A large fraction of these was also found to be differentially expressed in the comparison between wild-type Arabidopsis and the *fit1* mutant (Colangelo and Guerinot, 2004). The *fit1* mutant is defective in a bHLH transcription factor controlling several genes involved in iron deficiency response. When comparing the list of 72 genes of which the expression is (partially) dependent on *FIT1* (Colangelo and Guerinot, 2004) with the zinc excess-induced cluster (Table I; Supplemental Table S2), there are 30 genes in common. This apparent interaction between zinc and iron homeostasis in Arabidopsis, with zinc excess leading to iron deficiency, is not supported by a clear decrease in iron concentration in Arabidopsis leaves (Fig. 1), suggesting that this change in gene expression is indeed effective in avoiding actual iron deficiency in leaves.

For *T. caerulescens*, the zinc deficiency and zinc excess response is slightly different from Arabidopsis. This does not seem to be due to technical hybridization differences. The expression of the *T. caerulescens* genes confirmed by RT-PCR corresponded very well with the results obtained from the microarray analysis (Fig. 3; Supplemental Table S13). One cluster of coregulated genes is clearly differently expressed in *T. caerulescens* compared to Arabidopsis (Table III; Supplemental Table S5). Whereas in Arabidopsis the three ZIP family members *ZIP3*, *ZIP4*, and *ZIP9* are only more highly expressed under zinc deficiency, their *T. caerulescens* orthologs are also relatively highly expressed under sufficient zinc conditions. Based on sequence similarity, the *T. caerulescens ZNT1* and *ZNT2* genes appear to be the orthologs of the Arabidopsis *ZIP4* and *IRT3* genes. Both were previously found to be very highly expressed in *T. caerulescens*, almost regardless of the zinc concentration in the medium (Pence et al., 2000; Assunção et al., 2001). Also, under zinc deficient conditions, these two *T. caerulescens* genes are much more highly expressed than their Arabidopsis orthologs (Table IV; Supplemental Table S12).

Comparable to Arabidopsis, *T. caerulescens* expresses a cluster of genes in response to zinc deficient conditions (Table III; Supplemental Table S5), although this cluster is much smaller than in Arabidopsis. Such might be caused by differences in hybridization efficiency, but this is probably not the case, as the *T. caerulescens* orthologs of *FRD3*, *ZIP10*, and *HMA4* are not significantly differentially expressed within *T. caerulescens*, even though they are much more highly expressed in *T. caerulescens* than in Arabidopsis (Table IV; Supplemental Table S12). In a recent microarray study, *FRD3* and *HMA4* also appeared to be constitutively more highly expressed in *A. halleri* compared to Arabidopsis (Talke et al., 2006). Similarly, the expression of the *NAS2* and *FER1* orthologs in *T. caerulescens* is more or less constitutive rather than zinc deficiency induced as in Arabidopsis (Fig. 3).

The strong expression of *NAS2* in *A. halleri* compared to Arabidopsis (Weber et al., 2004; Talke et al., 2006) was not found in *T. caerulescens*. The different expression profiles between Arabidopsis and *T. caerulescens* of the other three *NAS* genes (Fig. 3) suggest a major function for these genes in the metal adaptation of *T. caerulescens*. The presence of at least four *NAS* gene copies in both species, which are apparently all functional and highly redundant (no visible phenotypes are observed in soil-grown single and double KO Arabidopsis mutants; data not shown), will have provided ample flexibility to sustain adaptive changes in *NAS* gene expression. Also, in view of the observed effect on, especially, nickel tolerance upon NAS overexpression in nonaccumulating, nontolerant species (Douchkov et al., 2005; Kim et al., 2005; Pianelli et al., 2005), the *NAS* genes may be crucial to metal tolerance in *T. caerulescens*.

Most interesting for the identification of genes that contribute to the adaptation of *T. caerulescens* to high zinc exposure are the genes that are differentially expressed when comparing *T. caerulescens* and Arabidopsis at comparable zinc exposures (Table IV; Supplemental Table S10). More than 2,200 genes are significantly ($P < 0.05$) differentially expressed (≥5-fold) at any of the three zinc exposure treatments. This compares well with the recent transcript profile comparison of *T. caerulescens* and *Thlaspi arvense* shoot tissue, in which close to 3,500 genes were found to be more than 2-fold differentially expressed at $P < 0.05$ (Hammond et al., 2006). More than 50% of the genes we find more highly expressed in *T. caerulescens* are of unknown function. In a recent *T. caerulescens* EST analysis (Rigola et al., 2006), especially genes of unknown function were overrepresented while genes involved in general and protein metabolism were underrepresented when compared to Arabidopsis. Even though the fraction of genes involved in (a) biotic stress response is comparable in both species, the stress response genes expressed in *T. caerulescens* are generally different from those expressed in Arabidopsis.

We further analyzed this large set of species-specific differentially expressed genes in different ways. When

sorting them according to the highest differential expression under zinc deficiency, which we consider to be most informative, there are several genes that are more than 100-fold higher expressed in *T. caerulescens*. Among the 15 most differentially expressed genes are four *PDF* or defensin genes, of which *PDF1.1* is close to 1,000-fold higher expressed under zinc deficient and excess conditions. The biological role of defensins is not very clear. These small Cys-rich peptides are generally induced by fungal infections and implicated in pathogen defense, hence their name (Thomma et al., 2002). Mirouze et al. (2006) recently showed that the *A. halleri PDF* family confers zinc tolerance, and they hypothesized that defensins interfere with divalent metal cation trafficking to confer the zinc tolerance phenotype. In Arabidopsis, expression of *PDF1.2* is induced by the stress hormone jasmonic acid (JA; Penninckx et al., 1998). Maksymiec et al. (2005) recently showed that heavy metal stress also induces JA accumulation in plants. Armengaud et al. (2004) found that *PDF1.2a*, *PDF1.2b*, *PDF1.2c*, and *PDF1.3* are also among the most induced genes upon potassium starvation in Arabidopsis, and they suggested a relation between potassium starvation and JA signaling. Of the total 415 genes they found to be differentially expressed under potassium starvation, we found 46 genes to be more highly expressed in *T. caerulescens* compared to Arabidopsis. How or if JA, potassium starvation, and zinc response are correlated remains elusive. However, in this context it is also interesting to note that *HAK5*, *KUP3*, and *KAT*, three potassium transporter genes, are much more highly expressed in *T. caerulescens* roots compared to Arabidopsis.

Zinc hyperaccumulation is a constitutive trait in *T. caerulescens* and, thus, we expect it requires a constitutive expression of metal hyperaccumulation genes and no specific induction at zinc deficiency or excess. It is complicated to identify such zinc accumulation genes from the large set of more or less constitutively higher expressed *T. caerulescens* genes, as many genes in this large set will be involved in general species differences. However, when considering the 16 most highly expressed genes at 100 μM $ZnSO_4$, already six metal homeostasis genes are among them, four of which are known zinc transporters: *HMA4*, *MTP1*, and the already discussed *ZIP4* and *IRT3*. *HMA4* was previously identified by Papoyan and Kochian (2004) as a zinc-transporting, P-type ATPase possibly involved in zinc hyperaccumulation, particularly in loading of zinc into the xylem. The *T. caerulescens* ortholog of the Arabidopsis *MTP1* gene was previously described as the *ZTP1* gene (Assunção et al., 2001) and has been suggested to play a role in metal tolerance of *T. caerulescens* (Assunção et al., 2001) and *Thlaspi goesingense* (Persans et al., 2001; Kim et al., 2004). Paralogs of the *AtMTP1* gene in *A. halleri* also cosegregate with zinc tolerance in a segregating population (Dräger et al., 2004). Other zinc transporter genes that are more highly expressed in *T. caerulescens* are *HMA3*, *MTP8*, and *NRAMP3*. *HMA3* is a P-type ATPase similar to *HMA4*. When expressed in yeast, it is able to transport cadmium, but zinc transport could not be proven and in Arabidopsis the expression of the gene is not affected by exposure to zinc (Gravot et al., 2004). The *MTP8* gene is another member of the cation diffusion facilitator family. Especially at zinc deficient and sufficient conditions, the gene is more highly expressed in *T. caerulescens* compared to Arabidopsis, suggesting a function in zinc uptake, although the regulation in Arabidopsis by *FIT1* (Colangelo and Guerinot, 2004) also indicates a role in iron homeostasis. An *mtp8* KO mutant was examined but not found to display any aberrant phenotype under differential zinc exposure (data not shown). *AtNRAMP3* is a vacuolar transporter that is able to transport iron and cadmium but not zinc (Thomine et al., 2000). The specific induction of *TcNRAMP3* gene expression by zinc deficiency and excess zinc suggests it plays an important role in mobilization of zinc and iron in *T. caerulescens* as in Arabidopsis (Lanquar et al., 2005).

Unexpected was that, in contrast to Arabidopsis, expression of the iron homeostasis genes *IRT1*, *IRT2*, and *FRO2* is not induced in *T. caerulescens* upon excess zinc treatment. When we tested with RT-PCR, we could not detect the expression of *TcIRT1* except in roots at lower zinc exposure levels (Fig. 3B; Supplemental Table S13). This suggests that *T. caerulescens* is either able to regulate zinc and iron homeostasis independently, unlike Arabidopsis, or that the continued expression of zinc transporters at high zinc exposure levels ensures low-efficiency, but sufficient, iron uptake in *T. caerulescens*. The *IRT1* gene plays an interesting role in metal homeostasis in *T. caerulescens*, since it is very highly expressed upon iron deficiency in the cadmium-hyperaccumulating accession Ganges but much lower in the cadmium-excluding accession Prayon (Lombi et al., 2002). The latter is physiologically and geographically very close to La Calamine.

Two metallothionein genes, *MT2B* and especially *MT2A*, are very highly expressed in *T. caerulescens*. MT2 expression is generally associated with copper stress tolerance (Zhou and Goldsbrough, 1995; van Hoof et al., 2001), but overexpression in *Vicea faba* also induced cadmium tolerance (Lee et al., 2004). Why these two, more copper-associated genes are so highly expressed in *T. caerulescens* is unclear. High zinc uptake due to high expression of zinc transporters with a low affinity for copper could of course cause some copper stress, but high copper levels have not been reported for *T. caerulescens*. It is more likely that these genes have a function in general stress response or, alternatively, serve to maintain copper homeostasis, as was also suggested for the *MT3* gene of *T. caerulescens* (Roosens et al., 2004).

Very surprising was the relatively high expression of 24 genes with a suggested function in lignin biosynthesis (Ehlting et al., 2005) and 13 genes implicated in suberin biosynthesis (*CER3*, *CER6*, and 11 LTP genes; Costaglioli et al., 2005) in *T. caerulescens* (Table IV). *CER3* is known to be expressed in Arabidopsis roots,

but the expression of *CER6* in *T. caerulescens* roots is very different from the expression in Arabidopsis (Hannoufa et al., 1996; Hooker et al., 2002). The high expression of lignin/suberin biosynthesis genes coincides well with the progressed U-shaped lignification/suberinization of the endodermis cells and the occasional presence of a second endodermis layer found in *T. caerulescens* roots, but not in Arabidopsis roots (Fig. 2). Casparian strip development and lignification in cortical cells also was recently observed by Zelko et al. (2005) in *T. caerulescens* but not in the closely related nonaccumulator *T. arvense*. Comparable endodermis wall thickenings were also observed in the salt-adapted crucifer *Thelungiella halophila* (Inan et al., 2004). Strong deposition of lignin and suberin on the radial and inner tangential walls resulting in a U-like appearance of the endodermal cells is not uncommon for plants (Zeier and Schreiber, 1998). Since this cell wall deposition occurs most prominently at older parts of the root where root hairs are no longer active, we hypothesize that this layer acts to prevent excess efflux of metals from the vascular cylinder rather than to prevent uncontrolled influx.

With so many genes differentially expressed, one also expects alterations in the transcript levels of transcription factors. In the *T. caerulescens*-Arabidopsis comparison, we found 131 transcriptional regulators with more than 5-fold higher expression (FRD $P < 0.05$) in *T. caerulescens* (Supplemental Table S14). Of 19 genes that are more than 10-fold higher expressed under zinc sufficient conditions, two genes (*INO* and *SPL*) are associated with flower development in Arabidopsis (Villanueva et al., 1999; Yang et al., 1999) and expression is very atypical. However, in line with this atypical expression, we also found the *FIS2* gene more highly expressed in *T. caerulescens* roots. In Arabidopsis, this gene is predominantly expressed in developing seeds (Luo et al., 2000), but also in *A. halleri* and *Arabidopsis lyrata* this gene is induced in roots in response to zinc exposure (www.genevestigator.ethz.ch).

In conclusion, the comparative transcriptional analysis of the hyperaccumulator *T. caerulescens* and the nonaccumulator Arabidopsis emphasizes the role of previously implicated zinc homeostasis genes in adaptation to high zinc exposure, but also suggests a similar role for many more, as yet uncharacterized genes, often without any known function. While some of these genes were also differentially expressed when comparing *A. halleri* with Arabidopsis, many more were not or at very different levels, suggesting that there will be overlap in the mechanisms of metal accumulation and metal tolerance but also many differences between metal hyperaccumulator species.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana Columbia-0 (Arabidopsis) and *Thlaspi caerulescens* J. & C. Presl accession La Calamine seeds were germinated on garden peat soil (Jongkind BV). Three-week-old seedlings were transferred to 600-mL polyethylene pots (three plants per pot) containing a modified half-strength Hoagland nutrient solution (Schat et al., 1996): 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 μM KCl, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 20 μM Fe(Na)EDTA. The pH buffer MES was added at 2 mM concentration and the pH was set at 5.5 using KOH. Each polyethylene pot contained three seedlings of *T. caerulescens* or Arabidopsis. Three weeks after growing on this solution, the *T. caerulescens* plants were transferred for 7 d to the same modified half-strength Hoagland nutrient solution with a deficient (0 μM), sufficient (100 μM), or excess (1,000 μM) $ZnSO_4$ concentration. The Arabidopsis plants were transferred to the same nutrient solution with deficient (0 μM), sufficient (2 μM), or excess (25 μM) $ZnSO_4$. During the first 3 weeks, the nutrient solution was replaced once a week and thereafter twice a week. Germination and plant culture were performed in a climate chamber (20°C/15°C day/night temperatures; 250 μmol light m^{-2} s^{-1} at plant level during 14 h/d [*T. caerulescens*] or 12 h/d [Arabidopsis]; 75% relative humidity; Assunção et al., 2001).

Root and Shoot Metal Accumulation Assay

Two pools of three plants, grown as described before, were used per treatment. After 4 weeks of growth, the plants were harvested, after desorbing the root system with ice-cold 5 mM $PbNO_3$ for 30 min. Roots and shoots were dried overnight at 65°C, wet-ashed in a 4:1 mixture of HNO_3 (65%) and HCl (37%) in Teflon bombs at 140°C for 7 h, and analyzed for zinc, iron, and manganese using flame atomic absorption spectrometry (Perkin-Elmer 1100B). Metal concentrations in roots and shoots were calculated as μmol per g DW.

Microarray Experiment

A common reference model was used to design the microarray experiment (Yang and Speed, 2002), in which cDNA from *T. caerulescens* roots exposed to 100 μM (sufficient) zinc was used as the common reference. Every slide was always hybridized with the common reference sample and with a sample from one of the treatments (Arabidopsis or *T. caerulescens* exposed to deficient, sufficient, or excess zinc). The common reference was labeled with the fluorescent dye Cyanine 3 and the treatment samples were labeled with Cyanine 5. As a quality-control step, we performed a dye-swap hybridization for one sample (from *T. caerulescens* roots exposed to sufficient zinc).

Roots of one pot containing three Arabidopsis or three *T. caerulescens* plants per treatment were pooled and homogenized in liquid nitrogen. Each pool was considered as one biological replicate and two biological replicates were used. Total RNA was extracted with Trizol (Invitrogen). Approximately 300 mg tissue was used for the RNA extraction performed according to the manufacturer's instructions. After the RNA extraction, total RNA was purified with RNeasy spin columns (Qiagen Benelux B.V.) and genomic DNA was digested with the RNase-free DNase set (Qiagen Benelux B.V.). Ten micrograms of total RNA was used to synthesize cDNA with MMLV reverse transcriptase and DNA primer. The cDNA was labeled with Cyanine 3-dCTP or Cyanine 5-dCTP and hybridized to Agilent Arabidopsis 3 60-mer oligonucleotide microarrays (Agilent Technologies) representing approximately 40,000 putative genes. The microarrays contain all Arabidopsis genes of known function and genes with a high degree of similarity to genes of known function from heterologous organisms. This includes more than 27,000 annotated genes and more than 10,000 nonannotated transcripts based on massive parallel sequence signature data (http://www.chem.agilent.com) and is thus an almost complete representation of the Arabidopsis transcriptome.

After hybridization, the slides were scanned, analyzed, and normalized with the Agilent Feature Extraction software (Agilent Technologies). The arrays were first normalized using Agilent's standard normalization within each array. The remaining statistical analysis was done using the limma package (Smyth, 2005a) in R/BioConductor (Gentleman et al., 2004). Between-array quantile normalization was performed on the common reference channel while leaving the log ratios unchanged (Yang and Thorne, 2003). To find differentially expressed genes, we performed a separate channel analysis (Smyth, 2005b) between the pairs of interest using a moderated *t* test (Smyth, 2004). This test is similar to a standard *t* test for each probe except that the SES are moderated across genes to ensure more stable inference for each gene. This prevents a gene from being judged as differentially expressed with a very small fold change merely because of an accidentally small residual SD. The resulting *P* values were corrected for multiple testing using the Benjamini-Hochberg FDR adjustment (Benjamini and Hochberg, 1995). Genes were

considered to be significantly differentially expressed if both the FDR *P* values were <0.05 (controlling the expected FDR to no more than 5%) and the fold change was ≥3 (within species) or ≥5 (between species). Genes found to be significantly differentially expressed were clustered using Cluster/Treeview (Eisen et al., 1998). Average linkage hierarchical clustering with uncentered correlation was used within Cluster to perform the clustering analysis. Primary microarray data are available in ArrayExpress E-MEXP-877.

Genomic DNA hybridizations were performed using 1 μg random primed genomic DNA. As a quality-control step, we performed a dye-swap hybridization. After hybridization, the slides were scanned, analyzed, and normalized with the Agilent Feature Extraction software (Agilent Technologies). The arrays were further normalized using linear Lowess analysis. The features that hybridized with Arabidopsis genomic DNA (both polarities of the dye swap) and did not hybridize with *T. caerulescens* genomic DNA were extracted from the dataset by Spotfire using a fold change ≥3 as the cutoff value.

Semiquantitative RT-PCR

Total RNA of leaves and roots of a third Arabidopsis or *T. caerulescens* biological replica was extracted with Trizol (Invitrogen). Selected *T. caerulescens* genomic and cDNA fragments were PCR amplified using primers designed for the orthologous Arabidopsis gene and cloned into the pGEM-T Easy vector (Promega). Clones were sequenced and new primers were designed for semiquantitative RT-PCR to ensure amplification of the correct *T. caerulescens* gene. Five micrograms of total RNA was used to synthesize cDNA with MMLV reverse transcriptase (Invitrogen) and oligo(dT) as a primer (Invitrogen). The PCR amplification was performed with a cDNA aliquot (1 μL) and gene-specific primers (Supplemental Table S13). Care was taken to design Arabidopsis primers at comparable positions and with comparable length and T_m as for *T. caerulescens* primers to allow proper comparison of the expression data. Primers for Tubulin (Supplemental Table S13) were used as a control for similar cDNA quantity between the samples. Between 25 to 35 PCR cycles (30 s at 94°C, 30 s at 50°C, and 60 s at 68°C) were performed in a 50-μL volume, preferably with the same number of cycles for Arabidopsis and *T. caerulescens* samples. Twenty microliters of the reaction was separated on an ethidium bromide-stained 1% agarose gel. Gel-image analysis using QuantityOne software (Bio-Rad) was used to quantify the DNA fragment intensities (Supplemental Table S13). The DNA fragment intensities were corrected for background signal and corrected for cDNA quantity using the intensities of Tubulin.

Microscopic Analysis of Lignification in *T. caerulescens*

T. caerulescens La Calamine seeds were germinated and plants were grown as described before on sufficient zinc medium. After 4, 6, and 9 weeks, roots of two plants were collected and hand sections were made by repeatedly chopping roots on a microscope slide using a razorblade. These sections were analyzed with a Nikon Labophot bright-field microscope.

T. caerulescens sequences used for semiquantitative RT-PCR analysis have been deposited with the EMBL/GenBank data libraries under accession numbers DQ384055 (*TcAPX2*), DQ384057 (*TcbHLH100*), DQ923700 (*TcCSD1*), DQ384056 (*TcFER1*), DQ384058 (*TcFRO4*), DQ923702 (*TcHAK5*), DQ384059 (*TcIRT1*), DQ384060 (*TcZIP1*), DQ384061 (*T. caerulescens* ortholog of prolyl oligopeptidase; At1g20380), DQ923699 (*T. caerulescens* ortholog of lipid transfer protein related; At1g27950), DQ923701 (*T. caerulescens* ortholog of Suc synthase; At4g02280), and DQ923703 (*T. caerulescens* ortholog of calcineurin-like phosphoesterase; At5g50400).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Arabidopsis genes more lowly expressed under zinc deficient conditions compared to sufficient and excess zinc.

Supplemental Table S2. Arabidopsis genes more highly expressed under excess zinc conditions compared to sufficient or deficient zinc supply.

Supplemental Table S3. Arabidopsis genes more highly expressed under zinc deficiency compared to sufficient and excess zinc supply.

Supplemental Table S4. Arabidopsis genes more lowly expressed under excess zinc compared to deficient and sufficient zinc conditions.

Supplemental Table S5. *T. caerulescens* genes more highly expressed under deficient compared to sufficient and excess zinc supply.

Supplemental Table S6. *T. caerulescens* genes more highly expressed under deficient and excess zinc conditions compared to sufficient zinc.

Supplemental Table S7. *T. caerulescens* genes more highly expressed under excess zinc conditions compared to deficient and sufficient zinc.

Supplemental Table S8. *T. caerulescens* genes more lowly expressed under zinc deficient conditions compared to sufficient and excess zinc.

Supplemental Table S9. *T. caerulescens* genes more lowly expressed under deficient and excess zinc conditions compared to sufficient zinc.

Supplemental Table S10. *T. caerulescens* genes more highly expressed under zinc deficiency, sufficiency, or excess compared to Arabidopsis.

Supplemental Table S11. Expressed genes in *T. caerulescens* for which no expression was detected in the roots of Arabidopsis under all three conditions tested.

Supplemental Table S12. A selection of genes more highly expressed in *T. caerulescens* compared to Arabidopsis.

Supplemental Table S13. Differentially expressed genes verified by semiquantitative RT-PCR.

Supplemental Table S14. Transcription factor genes more highly expressed in *T. caerulescens* compared to Arabidopsis.

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